# PERINATAL HIGH-FAT DIET AND BISPHENOL A: EFFECTS ON BEHAVIOR, GENE EXPRESSION AND NEUROANATOMY IN THE MEDIAL PREFRONTAL CORTEX

BY

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## ABSTRACT

Bisphenol A (BPA) is a synthetic industrial compound that is used to create polycarbonate plastics and epoxy resins. When BPA is consumed, most commonly through food and drink, it can act as an endocrine disruptor binding to estrogen-related, estrogen, androgen, and thyroid receptors. Abnormal binding of these receptors could alter normal brain development and cause lasting changes in gene expression, behavior and neuroanatomy. Additionally, perinatal HFD has been reported to cause long-term alterations in anxiety behavior and gene expression in the brain. Given that BPA and HFD are both ubiquitous in the environment and Western society, it is important to understand the possible effects and interactions on the brain. In the current study, I investigate the long-term impact of environmentally relevant exposure to BPA and a 45% fat diet during perinatal development. Zero (control), 40 or 400 μg/kg/day BPA was orally dosed to pregnant dams from gestational day 2 through birth, and then directly to each pup from postnatal day 1-10. The dams were fed either a control (15.8% kcal/fat) or a high fat diet (45% kcal/fat) during the same time period. After birth, the dams were observed for maternal care interactions with the litter. At postnatal day 10, tissue was collected from pups for gene expression and cytokine analysis. Littermates were observed for changes in periadolescent play behavior from postnatal day 26- 40, elevated plus maze (EPM) and social recognition in adulthood. Further, the cortex was collected in adulthood to assess gene expression, and the number of neurons, glia, synapses and microglia.

Significant alterations were found in maternal care, inflammatory markers, gene expression, play behavior and the elevated plus maze. Perinatal BPA exposure showed a trend toward a decrease in positive maternal care and increased the levels of inflammatory markers in the medial prefrontal cortex (mPFC) at P10, while perinatal HFD increased positive maternal

care and decreased the level of IL-6 in the mPFC at P10. Perinatal BPA exposure also altered periadolescent play behavior, with the control animals spending more time engaged in play behavior, the animals exposed to 40 μg/kg/day BPA spending the most time alone, and the animals exposed to 400μg/kg/day BPA spending the most time in passive contact. Additionally, perinatal BPA in the control diet groups produced anxiolytic behavior in males on the EPM, but did not affect female anxiety behavior. Perinatal HFD exposure did not alter the offspring's behavior in any of the tasks. Perinatal BPA and HFD both altered the gene expression of several hormone-related and pro-inflammatory genes. No significant changes were found in any of the measured aspects of the structure of the medial prefrontal cortex (number of neurons, glia, microglia, synapses, the volume of white matter under the prefrontal cortex and synapses per neuron). However, the pattern of the increases in the number of neurons and glia and the volume of white matter under the cortex was similar to those previously found by Sadowski et al. (2014). These results suggest that perinatal HFD and BPA generally do not have interactive effects, but perinatal BPA exposure does produce lasting effects on behavior, while structural effects in the medial prefrontal cortex are not robust.

*To my family and especially my daughter.*

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# CHAPTER 1: BISPHENOL A AND HIGH FAT DIET

## Basics of Bisphenol A

BPA is a synthetic industrial chemical used in the manufacturing of polycarbonate plastics. BPA, when condensed with a phosgene gas, creates carbonate linkages that make up the polycarbonate chain. The resulting polycarbonate polymers create strong, rigid plastics, which make BPA ideal for a wide range of consumer products (Krishnan et al., 1993). Items containing polycarbonate plastics include eyeglass lenses, dental sealants, medical devices, food storage containers, and water pipes. BPA is also a component of certain epoxy resins that coat the inside of the metal cans used for canned foods. The BPA based epoxy resins are used to keep the metal from corroding and contaminating food (Vandenberg et al., 2007). Concern over the safety of BPA has risen due to data suggesting that BPA is leaching out of these products. Despite its usefulness, there is evidence that environmental exposure to bisphenol A can have a negative impact on health and development.

Krishnan et al. (1993) discovered the leaching of BPA from polycarbonate flasks while searching for an estrogenic compound. BPA was competing with estradiol to bind at estrogen receptors on rat uterine tissue. Through this competition with estradiol for estrogen receptors, BPA was determined to be an endocrine disruptor. Endocrine disruptors are exogenous chemicals that are able to mimic or antagonize endogenous hormones and disturb normal functioning of the endocrine system. Since the endocrine system is involved in many aspects of development and daily functioning, disturbing this system could have drastic implications.

*Routes of Exposure*

Exposure to BPA has been linked to several sources, including landfill leachates (Kawagoshi et al., 2003), drinking water (Kleywegt et al., 2011) and air pollution (Loganathan & Kannan, 2011). However, the main route of exposure to BPA is consumption of food products that have been in contact with the polycarbonate plastics or epoxy resins. BPA has been found to migrate into canned foods and drinks exposed to the epoxy resin linings, including infant formula (Cao et al., 2009), vegetables (Brotons et al., 1995), dairy products (Kang & Kondo, 2003), soft drinks and beer (Cao et al., 2010). Migration of BPA into these foods increases with temperature and solution conditions. A higher amount of BPA was released into water samples from epoxy resins when cans were heated to 121 degrees Celsius compared to 105 degrees Celsius (Kang et al., 2003). BPA migration was also increased in 5% glucose solution, 5% sodium chloride solution, corn oil, olive oil or soybean oil when compared to water samples (Kang et al., 2003). Furthermore, Munguia-Lopez et al. (2002) found that storage time increases the amount of BPA migration from the epoxy resins when the cans are stored in the slightly increased temperature of 35 degrees Celsius, simulating a warm warehouse environment. These studies suggest that polycarbonate plastics and epoxy resins are leaching BPA contaminants into the environment and food products. Given this information, it is important to address both the health effects and the mechanisms of action of BPA once it enters the body.

*Pharmacokinetics*

The increased interest in the effects of BPA on human health has led to pharmacokinetic studies to determine the metabolism and bioavailability of BPA after exposure. Following oral administration (5mg) in humans, BPA is absorbed through the gastrointestinal tract and

conjugated with glucuronide by UDP- glucuronosyltransferase in the liver (Volkel et al., 2002), with the majority of BPA being transformed by the isoform UGT2B15 (Hanioka et al., 2008). The resulting metabolites, BPA-glucuronide (BPAG) and BPA-sulfate (BPS), are inactive forms (Snyder et al., 2000). Volkel et al. (2002) estimates that BPA is mostly eliminated from the body in 24 hours following consumption, with BPA metabolites being excreted through urine and the parent form being excreted through feces. The efficient clearance of BPA and its metabolites suggest that the major concern for human consumption is the consistent re-exposure to BPA, such as one would experience if eating canned goods multiple times per day.

The study by Volkel et al. (2002) is one of the few human studies investigating the pharmacokinetics of BPA. In order to extend the understanding of the mechanisms, many investigators have used animal models. A comprehensive study of BPA pharmacokinetics in rats determined that the route of administration results in significantly different BPA absorption, metabolism and bioavailability (Pottenger et al., 2000). BPA levels in the blood reached concentrations from 0 (not detectable) to 0.04μg/g in 15 minutes following 10mg/kg oral administration; whereas blood levels of BPA reached maximum concentrations of 0.69-0.87μg/kg in 15-30 minutes following 10mg/kg intraperitoneal administration and 0.34-0.39μg/kg in 15 minutes to 4 hours following 10mg/kg subcutaneous administration. The blood concentration levels suggest that route of administration leads to a difference in the amount BPA available to bind to and act on tissues. Oral administration shows the lowest bioavailability of BPA, suggesting a strong role of first pass metabolism on excretion. The majority of BPA is excreted through feces, 71-81% of the original dose with oral administration, 64-83% of original dose with intraperitoneal administration, and 54-80% with subcutaneous injection. The

remainder of the original dose is either secreted in urine or sequestered into tissues (Pottenger et al., 2000).

BPA was found to enter tissues, including adipose tissue, through unconjugated levels of BPA in the blood (Doerge et al., 2012). Animals dosed with oral administration retained 0.03% - 0.26% of the original dose in tissues. This was significantly lower than the tissue retention of 0.65%-0.85% of the original dose with intraperitoneal administration, and 1.03%-1.29% of the original dose with subcutaneous administration (Pottenger et al., 2000). Oral administration, which is the main route of exposure for humans, seems to have a high rate of clearance from the body, suggesting that humans may only have a small amount of BPA left in the body to act on tissues. However, BPA is so ubiquitous in the environment that human exposure is likely to include low doses several times per day.

*Levels of Exposure*

The Centers for Disease Control (CDC) provide continuous biomonitoring data from the United States population through the National Health and Nutrition Examination Survey. The most recent compiled data on BPA exposure levels is from 2011-2012. The total mean urinary concentration across all ages, sexes and ethnicities is 1.72 μg/g creatinine (1.58-8.24 μg/kg creatinine; $50^{th}$-$95^{th}$ percentile range) (CDC, 2015). It is not denoted whether or not the survey includes pregnant women. A separate study estimates the mean urinary level of BPA found in pregnant women to be 1.7 μg/g creatinine (estimated 1.0-11.0 μg/g creatinine; $5^{th}$ – $95^{th}$ percentile range) (Braun et al., 2011). The mean urinary concentration for pregnant women is identical to the mean found for the general population. Pregnant women characterize a special group when considering toxicants due to the sensitive developmental period of the fetus.

BPA levels in the placenta (and in some cases, the fetus) have been found to be higher than the mother's level (Schonfelder et al., 2002). Part of increased level may be due to the late expression of UDP-glucuronosyltransferase isoform UGT2B15. Human liver samples from 8 weeks gestation to 18 years were tested for the UGT2B15 isoform. UGT2B15 was not present in the fetal tissue until the third trimester, specifically gestational week 28 (Divakaran et al., 2014). Without UGT2B15 expressed in the fetal liver, the fetus is being exposed to BPA from the mother without a mechanism to conjugate the toxicant. The maternal-placental transfer of BPA is quite efficient and BPA can easily pass the placental barrier. After gestational weeks 28, the fetus displays Phase II metabolism and is able to conjugate the free BPA into BPAG and BPAS. These conjugates are unable to cross the placental barrier from the fetus back to the mother and accumulate in the amniotic fluid, which the fetus is continually ingesting. Recently, it was discovered in a chronically catheterized sheep model that the conjugate BPAG is deconjugated into the free BPA form in the fetus, creating a successive peak and trough exposure (Gauderat et al., 2016). The majority (70%) of the reconstituted BPA is eliminated from the fetus and amniotic fluid via maternal-placental transfer; however, it has been postulated that 30% of the reconstituted BPA is not eliminated and accumulates creating a constantly increasing baseline of free BPA concentration (Gauderat et al., 2016). Therefore, the developing fetus is being exposed to the BPA taken in by the mother.

*Effects on Humans*

Given the difficulty of tracking BPA ingestion during gestation and associating those effects with later behavior, few human correlational studies have been done. One meta-analysis correlated higher levels of gestational BPA exposure with later behavioral impairments between birth-12 years of age. Behavioral impairments included aggression, conduct issues,

hyperactivity, attention deficits, anxiety and depression (as reviewed in Ejaredar et al., 2016). The levels of gestational BPA exposure were based upon maternal self-report, gestational and childhood urine samples. Bisphenol A is only one of numerous environmental endocrine disruptors that would be present in the maternal urine, in addition to other events that could be interacting with or responsible for the children's behaviors. There is difficulty in referring causation from human studies as they are correlational out of necessity and have confounding factors; which makes animal models important for investigating both behavioral and neural effects.

For toxicological exposure in rat models, it is common to extend exposure through postnatal day (P) 10 to simulate human gestational development, since rats are born neurologically altricial compared to humans. At birth, the human brain undergoes a substantial growth spurt. This growth spurt is also observed in rats around P7 (Dobbing & Sands, 1979). Further, intracortical electrical activity via EEG in a newborn full-term human is most comparable to the electrical activity of a P12-13 rat (Romjin et al., 1991). Also considered for this comparison is synapse formation, glutamate decarboxylase and choline acetyltransferase development (Romjin et al., 1991) which all support the comparison between the third trimester in humans to the first ten postnatal days in the rat. Thus our study of BPA exposure will include both gestation and the first ten postnatal days.

## Non-human Studies of Perinatal BPA Exposure

### *Estrogen Receptors*

BPA is a weak endocrine disruptor, as it binds to classical estrogen receptors, estrogen receptor β (ERβ) with greater affinity than estrogen receptor α (ERα) (Kupier et al., 1997), but

with a 1/15,000 lower affinity for ERβ than estradiol (Gaido et al., 1997; Krishnan et al., 1993). However, there is another receptor that can also produce estrogenic effects: estrogen-related receptor gamma (Errγ), an orphan nuclear receptor that does not bind to estradiol. However, when Errγ is activated, the nuclear receptor can directly bind as a monomer to the estrogen response element (or half-site) and promote transcription, thereby eliciting indirect estrogenic effects (Hong et al., 1999; Heard et al., 2000). Interestingly, BPA has been found to bind with very high affinity to Errγ (Takayanagi et al., 2006). This finding, along with the high level of Errγ in the developing human fetal brain (Eudy et al., 1998) and human placenta (Takeda et al., 2009), indicate considerable opportunity for BPA to affect the prenatal brain. Furthermore, research suggests that BPA is a selective estrogen receptor modulator and can also block estrogen receptors (Kurosawa et al., 2002). Both mimicking and blocking estrogen could have deleterious effects at various action sites.

One effect of BPA is an increase or decrease of estrogen receptors in various structures of the brain. In a study investigating the effects of gestational exposure of BPA on male rats, pregnant dams were fitted with a subcutaneous osmotic pump that released 25μg/kg/day BPA, 250μg/kg/day BPA or a dimethylsulfoxide (DMSO) solution at a rate of 0.25μl/hour to G23 (day of birth) (Ramos et al., 2003). Gestational exposure to 25μg/kg/day BPA and 250μg/kg/day BPA was found to permanently increase the expression of mRNA for ERβ in the medial preoptic area (MPOA) in pubertal and adult male rats. While the increase in mRNA expression does not necessarily equate to increased amount of protein, there is a suggestion of an increase in the receptor protein. The increase in ERβ was not found in the medial basal hypothalamus, suggesting that BPA exposure has region-specific effects in the brain. Also, there was not an

increase in the expression of mRNA for ERα in either the MPOA or the medial basal hypothalamus, further suggesting that BPA is a selective modulator.

Cao et al. (2012) also studied the effects of BPA exposure on expression of ERα and ERβ in the MPOA and the medial basal hypothalamus in both female and male rats. Subcutaneous injections of 50μg/kg/day BPA were administered only from P0 to 2. Female pups were found to have a highly significant increased expression level of ERα over controls in the anteroventral periventricular nucleus (AVPV) on P4, which was, as expected, higher than the level of males. However, by P10, the expression level of ERα had fallen to equal levels with male expression of ERα. In the control animals, females had a higher expression level of ERα, suggesting that BPA has the ability to abolish the sex difference in the receptor levels in the AVPV. Further, reduction of expression levels of ERβ occurred in both males and females in the MPOA on P10 (Cao et al., 2012). In the mPFC, 0, 2, 20 and 200 μg BPA/kg/day orally during gestation caused sex-specific changes in estrogen receptors at P25 mice (Kundakovic et al., 2013). There was a sex-specific linear decrease in the relative gene expression of ERβ and a sex-specific quadratic change in the gene expression levels of Errγ. Errγ expression was U-shaped in females and inverted U-shaped in males (Kundakovic et al., 2013). These investigations suggest that acute exposure to BPA during critical brain development periods is sufficient to alter estrogen receptor expression levels in a sexually dimorphic fashion.

The results of these investigations find that BPA exposure at or below the US EPA's safe reference dose can produce significant changes in estrogen receptor levels at birth, puberty and adulthood. Furthermore, this survey of BPA on estrogen receptors highlights the lack of research on Errγ in the brain, which has been postulated to be the main receptor of BPA.

*Androgen and Thyroid Receptors*

BPA has also been found to bind to androgen and thyroid receptors, but the data are not as prolific as the studies on estrogen receptors. BPA binds to androgen receptors as an antagonist, and competes with [3H]5α-dihydroxytestosterone (DHT) as an androgen receptor ligand (Lee et al., 2003; Sun et al., 2006). DHT binding to androgen receptors is inhibited as much as 30-40% in the presence of BPA in ligand competition assays (Lee et al., 2003). Additionally, juvenile males exposed to 25μg/kg/day BPA during gestation had an increased level of testosterone in blood serum. The increased level of testosterone was transient and was not found in pubertal or adult males (Ramos et al., 2003). BPA interactions with androgen receptors during prenatal brain development could interfere with normal sexual differentiation.

Further, BPA also binds to thyroid receptors as an antagonist and can displace thyroid hormone from the receptor and activate co-activators or repressors (Moriyama et al., 2002). BPA can either activate or repress the transcription of thyroid-modulated genes, and can also affect the negative feedback loop of thyroid hormone on the pituitary gland (Zoeller et al., 2005). Thyroid hormone is critical in brain development as it plays a role in neural proliferation (Zoeller et al., 2002).

*Neural Effects*

Prenatal brain development is known to be sensitive to hormonal alterations. The most studied area of hormonal effects on brain development is in the hypothalamus, specifically in the nuclei in the MPOA: the sexually dimorphic nucleus (SDN) and the AVPV. The SDN is larger in males and programmed by perinatal estrogens (aromatized testosterone) in rodents. Shortly after birth, males and females undergo apoptosis in the SDN. Females lose a higher number of

neurons than males, who are protected from the same level of apoptosis by the neonatal estrogen programming in utero (Davis et al., 1996a). The AVPV is larger in females, with estrogen in neonatal/early postnatal life in male rats inducing apoptosis (Davis et al., 1996b). Both of these areas are susceptible to hormone disruption via endocrine disruptors. Perinatal BPA exposure has been found to abolish or even reverse the sex differences in these areas (McCaffery et al., 2013). The area of the SDN in adult males was significantly decreased with a prenatal dose of 10, 100, 1,000, or 10,000 μg/kg/day BPA, and the numbers of cells were significantly reduced in the 10 and 100 μg/kg/day BPA groups, abolishing the sex difference. Prenatal BPA did not have an effect on the volume or number of cells in the SDN of the female animals. However, the number of cells in the AVPV of the females was significantly reduced following prenatal exposure to 10, 100, and 10,000 μg/kg/day BPA. Interestingly, the number of cells in the AVPV of males was also reduced by 10 and 10,000 μg/kg/day prenatal BPA exposure (McCaffery et al., 2013).

The effects of BPA in the locus coeruleus have also been investigated. Pregnant female rats were exposed BPA in their drinking water from the first gestational day through P 21 that averaged to approximately 30μg/kg/day. In normal control rats, the volume and the number of neurons in the locus coeruleus are higher in females. However, BPA exposure was sufficient to reverse this sexual dimorphism (Kubo et al., 2003). Males exposed to BPA had a higher volume and a number of neurons in the locus coeruleus than females exposed to BPA. This was not simply an increase in the number of neurons in the males, as the females showed a reduction in neurons as compared to control numbers.

In the frontal cortex, perinatal BPA changes the levels of neurotransmitter metabolites (Honma et al., 2006). Dams were exposed to 0, 4 or 40 mg BPA/kg/day from G 6 to P20.

Three-week-old female offspring showed an increase in homovanillic acid (HVA; a dopamine metabolite), serotonin (5HT), 5-hydroxyindoleacetic acid (5HTAA; a 5HT metabolite) and HVA/dopamine ratio (HVA/DA) with 4 mg BPA/kg/day exposure. There was also an increase in the level of 3,4-dihydroxyphenylacetic acid (DOPAC; a dopamine metabolite) with 400 mg BPA/kg/day exposure. The levels of neurotransmitters changed with time as the 9 week old animals showed an increase in the level of norepinephrine in the forebrain, but a decrease in HVA/DA with 400 mg BPA/kg/day exposure. Likewise, there was a decrease in DOPAC with 40 mg BPA/kg/day exposure (Honma et al., 2006). Male offspring were not assessed in this study. Dopamine, norepinephrine and serotonin inputs to the mPFC are integral for cognitive functioning and alterations from endocrine disrupting chemicals are likely to interfere with behavior. However, it is important to note that the dose given in the Honma et al. (2006) study is in milligrams, a dose much higher than given in the majority of other studies and higher than the likely human exposure level (Taylor et al., 2011).

Normal neural development, including neurogenesis, neuronal migration, differentiation, and apoptosis occur during gestation and the early postnatal period making this time a sensitive period of brain growth that is especially susceptible to disruption. The vulnerability of the developing cortex has been highlighted through the introduction of exogenous substances during the gestational period, including the devastating results of alcohol (Scott-Goodwin et al., 2016), lead and polychlorinated biphenyls (Winneke, 2011). Sadowski et al.(2014) demonstrated the vulnerability of mPFC development to BPA exposure with the increase in the number of neurons and glia in adulthood following perinatal exposure. Dams were exposed to 0, 4, 40 or 400 μg BPA/kg/day via oral administration from the first day of gestation through parturition and then oral dosing of the pups from P1-10. A dose of 400 μg BPA/kg/day resulted in a higher number

of neurons and glia in the adult mPFC of male rats (Sadowski et al., 2014). This result was not observed in the female mPFC. A higher number of neurons in the cortex is a characteristic of male children with autism (Courchesne et al., 2007; Edmonson et al., 2014), and could contribute to alterations in behavior.

An increase in the number of neurons indicates that BPA is likely altering neurogenesis or apoptosis during development. Cortical neurogenesis begins around gestational day (G) 9 in rodents as progenitor cells divide and differentiate into neurons. The timing of neurogenesis and testosterone production in male fetuses overlaps during development, with neurogenesis peaking at E16/17 and testosterone production at E18 (Bayer, 1990; Weisz & Ward, 1980). Additionally, some neurons undergo apoptosis through the postnatal period. Currently there is no timeline for the apoptosis of neurons in the postnatal mPFC, but in the visual cortex it has been documented through P14 (Nunez et al., 2001). In our laboratory, we have evidence that it extends at least to P10 in the mPFC (Willing & Juraska, unpublished data). In addition, postnatal gonadal hormones have been shown to play a role in apoptosis in the rat visual cortex (Nunez et al., 2000). If apoptosis in the mPFC is also hormone-dependent, then the process could be vulnerable to endocrine disruptors such as BPA.

*Behavioral Effects of Perinatal Exposure*

BPA exposure below the US EPA's safe reference dose also can disrupt several types of behavior including maternal, social, and sexual behaviors. Acute administration of BPA during gestation and early postpartum can change maternal care. Della Seta et al. (2005) examined the effects of 40μg BPA/kg/day on several aspects of maternal care in rats. The day after mating, females began receiving BPA or vehicle control treatment through postnatal day 21.

Interestingly, only 53% of the BPA treated animals gave birth, whereas 78% of the control animals produced litters. While this is merely mentioned as a side note and not statistically analyzed, this difference may point to a disruption in reproductive success due to BPA exposure. For maternal behavior, BPA treated and control animals were analyzed on postnatal days 3, 4, 8 and 9. Thirty minutes before behavioral observation, the litter of pups was removed from the mother. At the beginning of the 30-minute observation period, four of the pups were reintroduced to the mother's cage at the opposite end from the nest. Frequency and duration of retrieving, anogenital licking, body licking, taking an arched-back posture, nursing, standing on nest and nest building were analyzed. A different four pups were used the following day. Della Seta et al. (2005) report a significant decrease in the overall duration of body licking of the pups by the BPA exposed mothers as compared to controls. The amount of maternal licking of the pups is known to influence later anxiety-related behaviors (Liu et al., 1997). The authors also report a marginally significant reduction in frequency of anogenital licking and duration of arched-back posture in nursing. A disruption of maternal care was also found in BALB/c mice with a dose-dependent U-shaped decrease and increase in frequency of licking, grooming and arched-back grooming with a maternal oral administration of 0, 2, 20 or 200 μg BPA/kg/day from G0 – parturition (Kundakovic et al., 2013).

The offspring (F1) of orally dosed pregnant female rats (F0) also show changes in behavior with either vehicle control or 40 μg BPA/kg/day from 10 days before dam (F0) mating through postnatal day 21 (Dessi-Fulgheri et al., 2002). After weaning, three same sex offspring were caged together, and no cage mates were siblings. The cage mates were placed in a neutral arena on postnatal days 35, 45, and 55. Female juvenile BPA-exposed rats displayed an increase in social play with other females when compared to controls, including pouncing and chasing the

other female cage mates. This increase in social play was equal to the level of social play by control males. The authors suggest that the low dose BPA exposure is making the normal female social behavior more male-like. The low dose BPA exposure also increased the level of social interest in males. Social interest included approaching male or female cage mates and general social orientation. Males also experienced a decrease in sociosexual exploration, which includes anogenital and body sniffing of females, and self-grooming. Female controls had a lower incidence of sociosexual exploration than control males, suggesting that the low dose BPA exposure lead males to exhibit slight female-like behavior that does not reach female levels of sociosexual exploration (Dessi-Fulgheri et al., 2002). These findings suggest that BPA exposure disrupts normal social and sexual juvenile behaviors.

Kubo et al. (2003) examined the effect of low dose BPA exposure on sexual and open-field behaviors. Pregnant female rats were exposed to BPA in their drinking water at approximately 30μg/kg/day from the first gestational day through postnatal day 21. The offspring were tested at 6 weeks of age in an open-field chamber that recorded the total distance traveled, time in the center of the chamber, and number of rearing episodes. Male rats exposed to BPA displayed an increase in the number of rearing episodes compared to control males, suggesting an increase in female-like behavior as control females have a higher number of rearing episodes than control males (Kubo et al., 2003). No other aspects of the open-field behavior were significantly altered.

Further, Kubo et al. (2003) also analyzed sexual behaviors in both the male and female offspring at 11-12 weeks of age. In males, sexual behavior was coded for number of mounts, intromissions, intromission rate (intromissions/mounts), latency to ejaculation, and duration from first mount to ejaculation. The only sexual behavior in males that was significantly altered was a

reduction in intromission rate. Female sexual behaviors, including number of ear wiggles, lordosis postures, and rejections, were not significantly altered by a perinatal exposure BPA. These results suggest that while Dessi-Fulgheri et al. (2002) found differences in sociosexual behavior in juveniles, low dose BPA exposure does not affect sexual behavior of adult rats other than the intromission rate of males.

The effects of BPA exposure on anxiety behaviors are commonly studied and often contradictory. Gestational BPA exposure of 0.4 to 4 mg/kg/day caused an increase in anxiety-like behaviors in male and female mice tested on the open field task, light/dark box and EPM (Xu et al., 2011). In another study, juvenile mice exposed to gestational BPA (averaged 150 μg/kg/day in maternal diet) spent less time on the distal portion of the open arm of the EPM, suggesting an anxiogenic effect of BPA (Cox et al., 2010). However, perinatal BPA exposure at 2.5, 25, or 2500 μg/kg/day did not have any effects on anxiety-like behavior in adolescence or adulthood in the open field task, EPM or zero maze in either male or female rats (Rebuli et al., 2015). And further, 100 μg/kg/day perinatal BPA exposure in male and female mice produced an anxiolytic effect in the open field task and 500 μg/kg/day produced an anxiolytic effect on the EPM (Tian et al., 2010). These are examples of the many conflicting reports regarding anxiety behavior following perinatal BPA exposure. Furthermore, there are reports of sex-specific alterations in anxiety behavior due to BPA exposure. For example, prenatal BPA exposure increased the number of entries and time into the open arms in male rats, suggesting a decrease in anxiety, while female offspring showed a decrease in the total number of entries into the arms suggesting a decrease in activity (Farabollini et al., 1999). The effects of BPA exposure on anxiety behavior seem to be heavily dependent upon species and BPA dosage.

Research addressing the cognitive effects of perinatal BPA exposure is also not conclusive. Our laboratory did not find a significant change in reference or working memory in adult male or female rats following 4, 40 or 400 μg/kg/day perinatal BPA exposure as tested in the 17-arm radial maze (Sadowski et al., 2014). Spatial memory was also tested in mice after perinatal exposure to 2 or 200 μg/kg/day BPA. The animals did not show a change in spatial memory as tested by the radial arm maze or Barnes maze (Ryan & Vandenbergh, 2006). However, working memory as assessed by the alteration behavior in a Y-maze was impaired in adult male and female mice following perinatal exposure to 100 or 500 μg/kg/day (Tian et al., 2010). The same study also found impairment in novel object recognition memory following 100 μg/kg/day (Tian et al., 2010). Perinatal BPA exposure of 40 μg/kg/day in Wistar rats also produced impairments in short term and long-term memory in the step-down avoidance task and latency to reach the platform in the Morris water maze in adulthood (Goncalves et al., 2010); however, another study of perinatal BPA exposure failed to cause disruption in memory in the Morris water maze (Jones & Watson, 2012). Variability in results across studies may be due to dose, species, and type of task. Still, these studies suggest that even BPA exposures below the safe reference dose set by the US EPA can produce disruptions in normal behavior.

*BPA and Inflammation*

Exposure to BPA has been shown to induce inflammation in the periphery and the brain. At least a 30% increase was found in pro-inflammatory cytokines, IL-12p70, IL-1α, IL-1β, and TNFα, in the peripheral serum of adult male mice perinatally exposed to BPA (Holladay et al., 2010). Another study found an increase in pro-inflammatory cytokines IL-6 and TNFα, and an increased activation of microglia in cortical tissue following perinatal BPA exposure (Luo et al., 2014). The BPA-induced increases in inflammation during development could contribute to

behavioral impairments in adulthood. In humans, gestational and neonatal exposure to inflammation results in a higher likelihood of developmental disorders, anxiety and depression, and cognitive impairments to memory, learning, and attention (as reviewed in Bilbo & Schwarz, 2009). Given the ubiquitous existence of BPA and the documented effects of inflammation on brain and behavior, clearly understanding the impact of BPA exposure on inflammation is critical.

## High Fat Diet

Effects of gestational HFD exposure on brain and behavior have not been investigated in nearly as much detail as BPA. However, one-third of pregnant women are obese and this is likely due to a HFD and overconsumption (King, 2006). The typical American diet is approximately 35% fat, with a healthy level of fat consumption approximately 20-25% (Freedman, 2001). Relatively recently, gestational HFD has been found to alter aspects of the brain and program offspring behavior (Bilbo & Tsang, 2010).

Gestational HFD with 60% fat content has been found to cause an increase in pro-inflammatory cytokine expression of cytokine, IL-1β, and microglial activation in the hippocampus of adult male and female offspring (Bilbo & Tsang, 2010); however, these increases did not necessarily correlate with impaired behavior. Interestingly, the male and female offspring from the dams fed a 60% fat HFD performed better in the Morris water maze, whereas the male offspring were more anxious on the EPM (Bilbo & Tsang, 2010). The increase in anxiety-like behavior with gestational HFD with 32% fat content has been found in other animals, including female non-human primates (Sullivan et al., 2010), where they also found alterations in the serotonergic system of the male and female offspring (Sullivan et al., 2010). Further, a maternal HFD with 60% fat content increased the level of IL-6 in a frontal sample of

an adult male offspring brain, and caused impairment in retention of the Morris water maze (White et al., 2009).

The studies investigating the effects of gestational HFD on offspring behavior suggest that inflammation plays a role in perinatal programming of the offspring's brain. Consumption of a HFD puts the periphery in a state of chronic inflammation (as reviewed in Bolton & Bilbo, 2014) and likely affects the fetus during the sensitive period of development.

*Research plan*

BPA and HFD have both been shown to increase inflammation in animal models (Luo et al.2014; White et al, 2009; Holladay et al.2010). However, no research has been conducted assessing the possible interactive effects of BPA and HFD despite the prevalence of endocrine-disrupting chemicals in our environment and high-fat diets in Western society. Further, previous data from our lab show an increase in the number of neurons and glia in the medial prefrontal cortex (mPFC) of male rats following perinatal exposure to 400 ug/kg/day of BPA (Sadowski et al., 2014). An increase in neurons in the prefrontal cortex is a characteristic of male children with autism (Courchesne et al., 2011; Edmondson et al., 2014) and there are suggestions that environmental endocrine disruptors, including BPA, could be affecting normal neural development (Ling et al., 2016). The aim of the following studies is to assess the impact of concurrent exposure to BPA and HFD on maternal care and offspring gene expression and cytokine levels as well as long-term social and anxiety-like behaviors (Fig. 1.1). In addition, the work of Sadowski et al.(2014) showing a significantly higher number of neurons and glia in the mPFC in male animals exposed to 400 µg/kg/day of BPA will be expanded on through examination of the number of synapses and synapses per neuron. Further, the number and

morphological state of microglia, which indicates degree of inflammation, will be analyzed. Doses of 40 and 400 μg/kg/day BPA are used because 40 μg/kg/day is below the Environmental Protection Agency (EPA)'s safe daily intake level (50 μg/kg/day), and 400 μg/kg results in plasma BPA levels similar to human exposure levels (Taylor et al., 2011). Additionally, our laboratory has previously found effects on the number of neurons and glia following exposure to these doses (Sadowski et al., 2014).

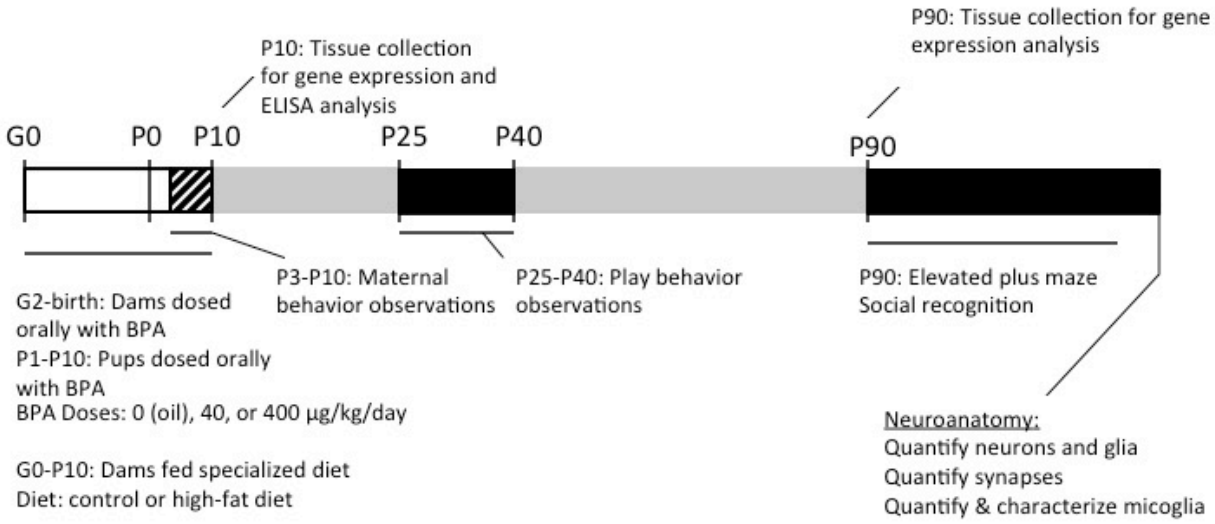



Figure 1.1. The timeline showing the following studies covering the behavioral, cellular and genetic effects of perinatal BPA and HFD exposure.

# CHAPTER 2: EFFECTS OF PERINATAL BISPHENOL A AND HIGH FAT DIET ON MATERNAL BEHAVIOR AND P10 INFLAMMATORY MARKERS IN THE MEDIAL PREFRONTAL CORTEX

The early environment, and specifically maternal care, can have long-lasting effects on the cellular composition (Liu et al., 1997) and behavior (Caldji et al., 1998) of rodent offspring with broad generalization to other mammalian species (Sanchez, 2006; McGowan et al., 2009). Offspring of mothers that displayed a high amount of licking and grooming of the pups have an increase in the expression of glucocorticoid receptors in the hippocampus, a reduction in the level of corticotropin-releasing hormone mRNA in the hypothalamus, and a decrease in the plasma levels of adrenocorticotropic hormone and corticosterone in response to restraint stress (Liu et al., 1997). Further, the offspring of mothers that had a high level of licking and grooming behavior also demonstrated a reduction in anxiety-like behavior in the open field and novel environment paradigms (Caldji et al., 1998). These studies exemplify the impact of early environment in shaping the brain and behavior in the offspring. Research in animal models has suggested that endocrine disruptors and dietary intake can each alter maternal behavior and expression of cytokines in the offspring, which may have implications for humans given the ubiquitous exposure of endocrine-disrupting chemicals in our environment and high-fat diets in Western society.

In Sprague Dawley rats, 40 μg/kg/day of BPA during gestation and lactation is sufficient to reduce the dams' time spent licking the anogenital area and body of the pups and nursing in an arched-back position (Della Seta et al., 2005). Further in Balb/c mice, 2, 20, and 200 μg/kg/day of BPA resulted in a dose-dependent U-shaped change in the time dams spent licking and grooming the pups, as well as nursing in the arched-back position (Kundakovic et al., 2013). These studies suggest changes in typical maternal care with concurrent BPA exposure.

However, it should be noted, Boudalia et al. (2014) did not find any significant effects of 5 μg/kg/day of BPA in maternal behavior in the Wistar rats treated during gestation and lactation, but the offspring showed a decrease in the time spent nursing and the percent of animals displaying the active arched nursing position.

Another factor that has been shown to affect maternal care is high-fat diet (HFD). Currently, one-third of pregnant women are obese and this is likely due to a HFD and overconsumption (King, 2006). Yet, few studies have investigated the implications of a HFD on concurrent maternal behavior. In Wistar rats, a HFD (45% kcal fat) during gestation and lactation resulted in a decrease in the percent of time dams licked and groomed their pups, but did not change the percent of time in contact with the nest (Connor et al., 2012), suggesting that HFD has a negative impact on maternal care. However, a study in Sprague Dawley rats fed a HFD (60% kcal fat) during gestation and lactation found an increase in time the dams spent nursing and in the arched-back nursing position. Further, the dams fed a HFD spent less time resting and more time caring for the pups (Purcell et al., 2011). Thus, the current research is divided on the impact of HFD on maternal care, but both studies suggest that HFD changes maternal behavior, which will have an impact on the offspring.

Pro-inflammatory cytokines in the brain are increased by perinatal BPA or HFD separately. Perinatal BPA exposure increases the protein level of cytokines, interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) in the frontal cortex of juvenile mice (Luo et al., 2014), which suggests that BPA during gestation and lactation can cause inflammation in the brain. Similarly, offspring from dams fed a HFD had a marked increase in protein expression of cytokine, IL-1β in the hippocampus at P20 and in adulthood (Bilbo & Tsang, 2010).

Inflammation in cortical areas could impact the cell populations and lead to cognitive effects; however, the possible interactions of BPA and HFD have yet to be studied.

Even with the prevalence of both factors in the human population, and the previous studies showing separate effects of HFD and BPA, the possible additive or interactive effects of HFD and BPA exposure on maternal care and offspring brain inflammation have not been investigated. The current study examines the possible interactive effects of BPA exposure and HFD during pregnancy and early postnatal period on concurrent maternal behavior and on a survey of pro-inflammatory cytokines in the mPFC of P10 offspring. The survey of cytokines includes TNF-α, transforming growth factor-β (TGF-β), monocyte chemoattractant protein (MCP)-1, IL-1α, IL-1β, IL-6, IL-15, and vascular endothelial growth factor (VEGF).

## Methods

### *Breeding*

Male and female Long Evans hooded rats (n=58 of each sex, Harlan Laboratories [now Envigo], Indianapolis, IN, USA) were housed in our vivarium for at least one week before being paired for breeding. They were kept on a 12:12 light/dark cycle and were allowed food and water ad libitum. Precautions were taken to reduce the environmental exposure to endocrine disruptors. All animals were housed in BPA-free polysulfone cages, watered with reverse osmosis filtered water in glass bottles, fed a low phytoestrogen food (Harlan 2020X; Teklad Diets, Madison, WI, USA) and exposed to a specialized diet during gestation and through P10. Breeding pairs were placed in suspended wire bottom cages and checked daily for the presence of sperm plugs. The day a sperm plug was detected was recorded as G0 and the dams were singly housed in a polysulfone shoebox cage. The breeding animals were paired for a maximum

of six nights. If no sperm plug was detected after six nights, the male was removed and another male introduced. From the first day of pregnancy, each female was assigned to one of six groups. The N for the control diet and BPA exposures are: 0 µg BPA/kg = 8; 40 µg BPA/kg = 10; 400 µg BPA/kg = 10. The N for the high-fat diet and BPA exposures are: 0 µg BPA/kg = 10; 40 µg BPA/kg = 10; 400 µg BPA/kg = 10.

*BPA Dosing*

BPA was suspended in tocopherol-stripped corn oil at 0, 0.1 mg BPA/ml, or 1.0 mg BPA/ml in order to administer 0 (control), 40 µg BPA/kg , or 400 µg BPA/kg respectively. To dose the adult dams, the required amount (0.4 µl/g body weight) was pipetted onto ½ of a cookie (Newman's Own organic alphabet cookie, vanilla flavor) and given to the animals. The animals readily consumed the cookie making this route of exposure non-stressful and similar to human ingestion of BPA. On G0 and G1, the dams were given ½ of a cookie with 0.4 µg/g tocopherol-stripped corn oil. Starting on G2 through parturition, the dams were given the cookie with the assigned BPA dose. The day of birth was recorded as P0 and the litters were not disturbed. Then daily from P1-10, each pup was individually dosed via pipetting the assigned solution (same as dam) directly into its mouth because lactational transfer of BPA is very low (Doerge et al., 2010). The 40 and 400 µg/kg doses were chosen because 40 µg/kg is below the EPA's safe daily intake level and 400 µg/kg results in plasma BPA levels similar to human exposure levels (Taylor et al., 2011). Additionally, our laboratory has previously found effects on the number of neurons and glia following exposure to these doses (Sadowski et al., 2014).

*Diet*

Starting on G0 through P10, the dams were fed either a control diet (CON; 15.8% kcal fat; D10012G, Research Diets Inc., New Jersey, USA) or a high-fat diet (HFD; 45% kcal fat; D12451, Research Diets Inc., New Jersey, USA; Table 2.1). The specialized diets were isocaloric for protein. The animals were allowed to feed Ad libitum. Number and sex ratio of the pups and daily body weight for dams and pups were recorded.

Table 2.1 Formula of CON and HFD

| | **CON** | **HFD** |
|---|---|---|
| **Macronutrients** | **% kcal** | **%kcal** |
| **Protein** | 20.3 | 20 |
| **Carbohydrate** | 63.9 | 35 |
| **Fat** | 15.8 | 45 |
| | | |
| **Ingredients** | **kcal** | **kcal** |
| **Casein, 30 Mesh** | 800 | 800 |
| **L-Cystine** | 12 | 12 |
| **Corn Starch** | 1590 | 291 |
| **Maltodextrin** | 528 | 400 |
| **Sucrose** | 400 | 691 |
| **Soybean Oil** | 630 | 225 |
| **Lard** | 0 | 1598 |
| **Vitamin Mix** | 40 | 40 |
| | 4 kcal/gm | 4.73 kcal/gm |

*Maternal Behavior Observations*

The dams were observed with their litters during the dark cycle from P3-10 using night vision goggles by an observer blind to treatment group. The dams' behavior was observed and recorded every 3 minutes for 90 minutes (30 observations per night). The recorded behaviors were nursing, licking of pups, pup retrieval, nest building, and being away from the nest. When

nursing and licking of pups occurred simultaneously, licking was preferentially recorded. Pup retrieval and nest building were rarely observed and therefore not included in the analyses.

*ELISA assays*

On P10, one male and one female from each litter was euthanized via CO2 and the brain was immediately removed, snap frozen in liquid nitrogen, and stored in a -80C degree freezer. The mPFC was excised from the frozen tissue. One hemisphere was used for ELISA assays. The second hemisphere was reserved for gene expression analysis (Chapter 4). A pre-coated ELISA plate (catalog #: EA-1501; Signosis, Santa Clara, CA) was used to analyze the levels of cytokines. Each plate can bind and measure TNF-α, TGF-β, MCP-1, IL-1α, IL-1β, IL-6, IL-15, and VEGF simultaneously, allowing for a survey of inflammatory factors. One hemisphere of theP10 PFC was lysed following manufacturer's instructions. Briefly, 1 ml of cell lysis buffer/100 mg of tissue was homogenized on ice. The lysate was centrifuged to separate the tissue from the supernatant, which was then diluted to 100 µg/ 100 µl per well and used for the protein analysis. The samples were run in duplicate and incubated in the plate overnight followed by three washes. A biotin-labeled antibody (specific to each cytokine) was added and allowed to incubate for 4 hours followed again with three washes. Next, a streptavidin-HRP conjugate was added to each well, incubated for 45 minutes, and followed with three washes. Finally, HRP-substrate was added and allowed to incubate for 30 minutes before the addition of stop solution to each well. The optical density of each well was determined with a microplate reader at 450 nm within 5 minutes.

*Statistical Analyses*

IBM SPSS Statistics (version 24) was used to conduct all analyses. A 3 (treatment) x 2 (diet) repeated measures linear mixed model with a first-order ante-dependence covariance structure (determined by small Akaike's Information Criterion) was used to analyze the growth curves for the dams' gestational body weights. 3 (treatment) x 2 (diet) ANOVAs were used to analyze litter size and sex ratio, as well as each maternal behavior (nursing, licking, and time away from nest). A 3 (treatment) x 2 (diet) x 2 (sex) ANOVA was used to analyze the difference in pup weights at both P1 and P10. To analyze the ELISA assays, percent change from the control group (control diet/0 dose BPA) was calculated for each cytokine, and then a 3 (treatment) x 2 (diet) x 2 (sex) ANOVA with plate number as a co-factor was used to analyze the percent change for each cytokine. LSD was used for post hoc analysis. When analyzing the effects of BPA treatment, each dose was only compared to the control group.

## Results

Neither BPA nor HFD had a significant effect on gestational body weights, litter size, or sex ratio (Fig. 2.1). There were also no significant differences in offspring body weights at P1; however, differences emerged by P10. There was a significant main effect of BPA treatment, $F(2, 106) = 5.48$, $p=.005$, with the 40 μg BPA/kg weighing less than control ($p=.002$; Fig. 2.1D). There was no effect of HFD or sex on the body weights of the P10 pups.

There was a significant decrease in the amount of time the HFD dams spent away from the nest compared to CON dams; $F(1, 52) = 0.25$, $p=.016$ (Fig. 2.2). But, there was no effect of BPA treatment and no interaction. HFD also marginally increased the amount of time the dams spent nursing the pups, $F(1, 52) = 3.84$, $p=.055$ (Fig. 2.2). There was no effect of BPA treatment

and no interaction. BPA had a marginal effect on licking behavior, $F(2, 52) = 3.03$, $p=.057$ (Fig. 2.2), with the 400 µg BPA/kg exposure group spending less time licking the pups than the control ($p=.055$). There was no effect of diet and no interaction.

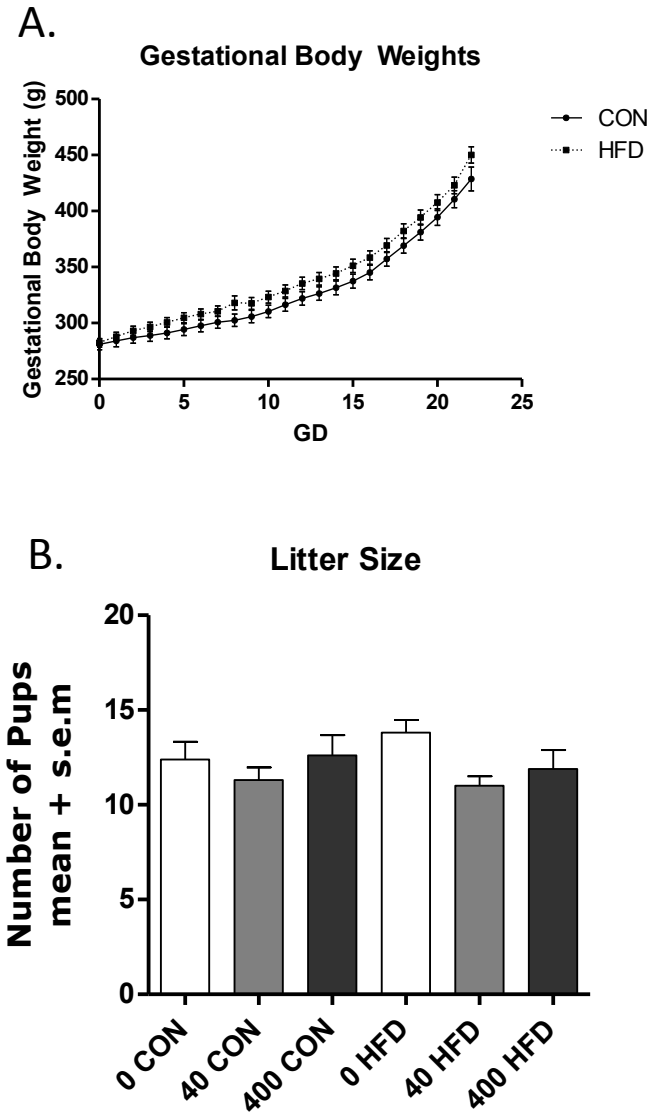



Figure 2.1. A. Gestational body weights of dams fed control (CON) or high fat diet (HFD). There were no significant differences between diets or BPA treatments. B. The size of the litters was not significantly different between diet and treatment. C. The sex ratio of the litters was not different between diet and treatment; >1 indicates a higher number of males. D. The body weights of the pups were not significantly different at P1, but at P10, a main effect of BPA treatment emerged. There were no significant differences in body weights between CON and HFD pups. (figure continued on next page)

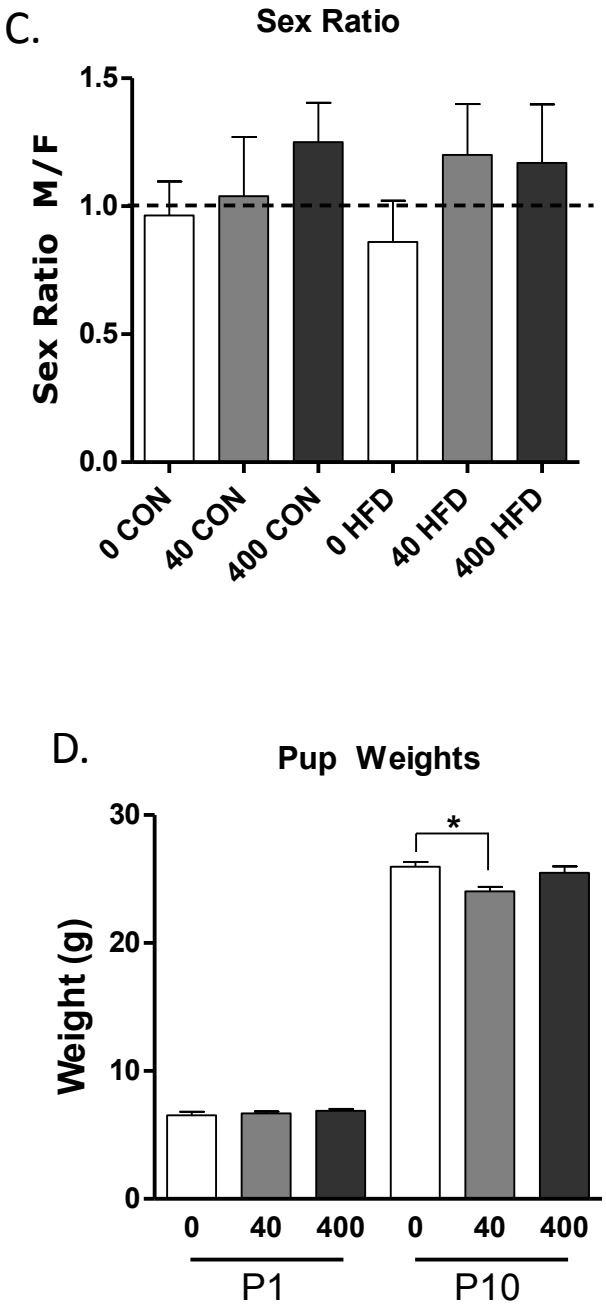



Figure 2.1. (cont.) A. Gestational body weights of dams fed control (CON) or high fat diet (HFD). There were no significant differences between diets or BPA treatments. B. The size of the litters was not significantly different between diet and treatment. C. The sex ratio of the litters was not different between diet and treatment; >1 indicates a higher number of males. D. The body weights of the pups were not significantly different at P1, but at P10, a main effect of BPA treatment emerged. There were no significant differences in body weights between CON and HFD pups.

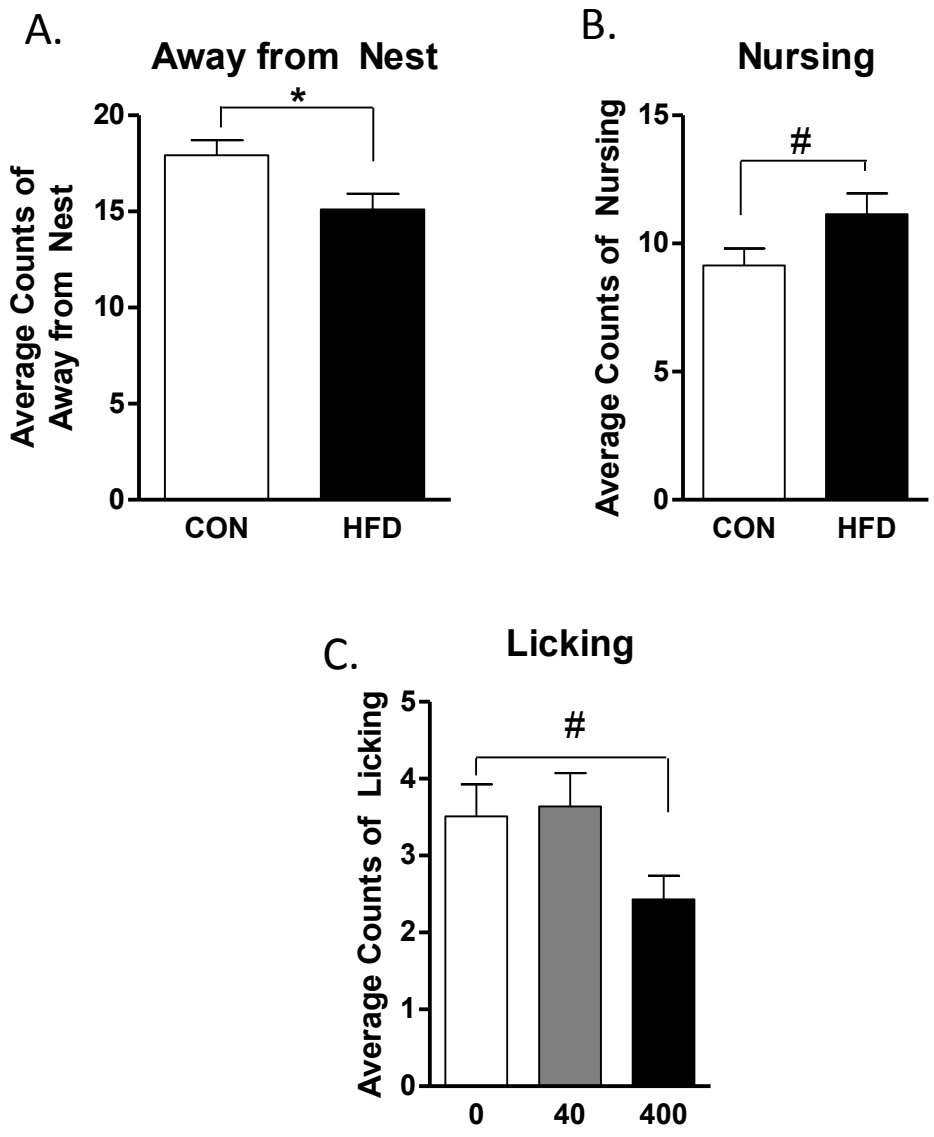



Figure 2.2. A. Dams exposed to CON diet spent more time away from the nest compared to the HFD exposed dams, *p<.05. There was no effect of BPA treatment on time spent away from nest. B. Dams exposed to HFD spent more time nursing the pups, #p<.06. There was no effect of BPA exposure on nursing behavior. C. Dams exposed to 400 μg BPA/kg spent less time licking the pups than control dams. There was no effect of diet on licking behavior.

The results of the ELISA assays are in Table 2.2. There were several instances of sex differences in the percent change compared to control animals (CON diet/ 0 dose) in cytokine levels in the mPFC following perinatal BPA and HFD exposure. Males showed a higher percent change from control in the level of cytokines TNFα, TGFβ, MCP-1, IL-1α, IL-15, and VEGF. Females had a higher percent change from control in the level of IL-6 cytokine. The only cytokine that did not show a sex difference was IL-1β. In addition to the sex difference, there was a significant main effect of diet on IL-6, $F(1,70)=11.25$, $p=.001$ (Fig. 2.3). HFD did not affect any other cytokines. There were no main effects of BPA on the levels of cytokines in the mPFC of P10 pups, but there were significant interactions between sex and BPA exposure in TNFα ,$F(2,68)=4.09$, $p=.021$, MCP-1 $F(2,65)=5.93$, $p=.004$, and VEGF, $F(2,68)=6.81$, $p=.002$ (Fig. 2.4) LSD posthocs found that males, but not females, had increased levels of TNFα, MCP-1 and VEGF following BPA exposure. Males had a significant increase in TNFα following 40 and 400 µg BPA/kg compared to control ($p=.021$ and $p<.001$, respectively). Males also had a significant increase in the levels of MCP-1 and VEGF following 400 µg BPA/kg exposure compared to controls ($p=.005$ and $p=.002$, respectively).

In order to assess the possible associations between maternal behavior and cytokine levels of the offspring, a two-tailed Spearman's correlation was conducted comparing maternal licking to each protein. Maternal licking was negatively correlated with levels of IL-1α ($r= -.257$, $p<.02$), IL-15 ($r= -.243$, $p<.03$) and VEGF ($r= -.249$, $p<.02$; Fig. 2.5). The remaining cytokines were not significantly correlated with maternal licking.

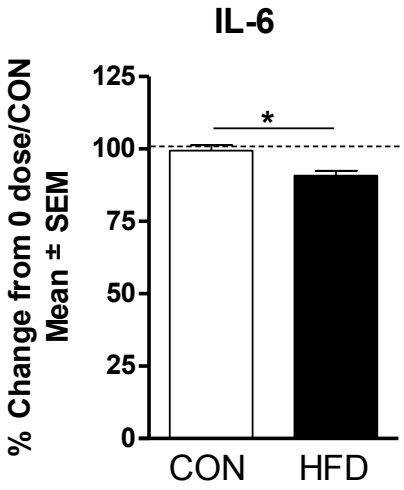



Figure 2.3. Protein concentration of IL-6 in mPFC. HFD resulted in a significant decrease (*p=.001) in the level of IL-6.

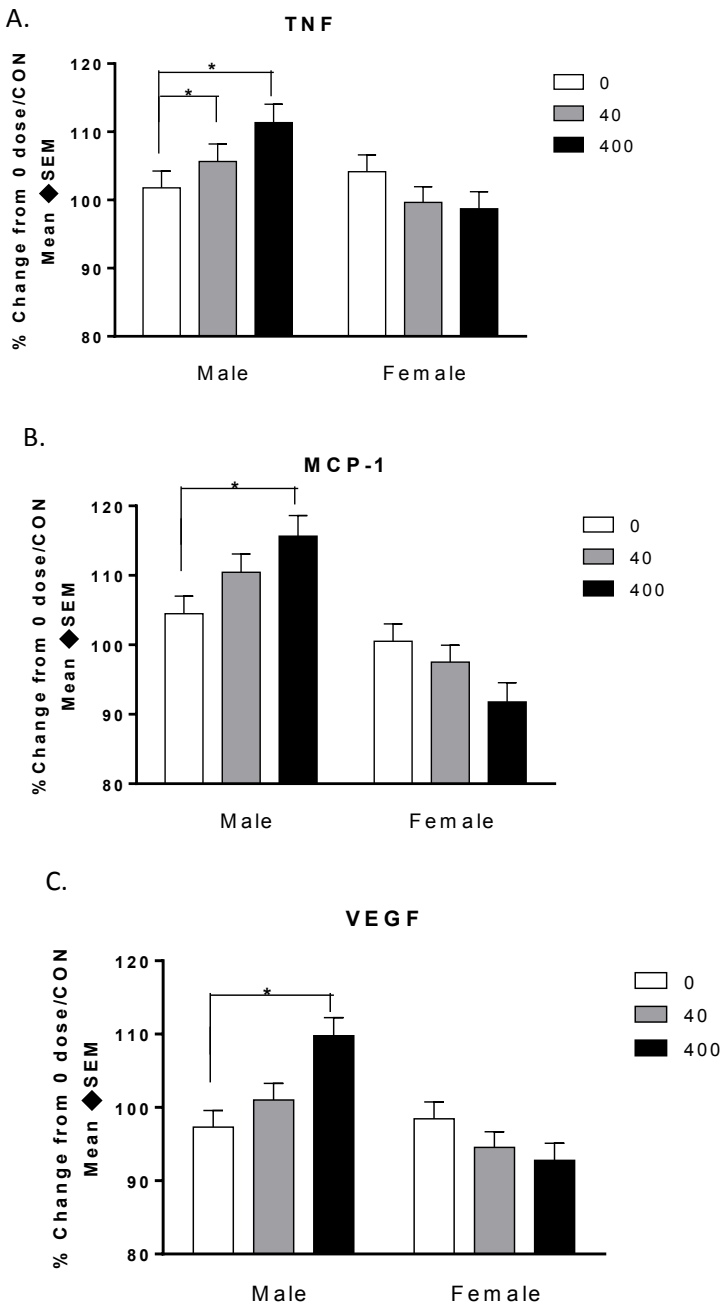



Figure 2.4. Males had significant increases in the levels of cytokines in the mPFC at P10 following perinatal BPA exposure. A. Male animals exposed to 40 and 400 µg/kg/day BPA had higher levels of TNFα than control animals. B. Male animals exposed to 400 µg/kg/day BPA had higher levels of MCP-1 than control animals. C. Male animals exposed to 400 µg/kg/day BPA had higher levels of VEGF than control animals, *p<.05.

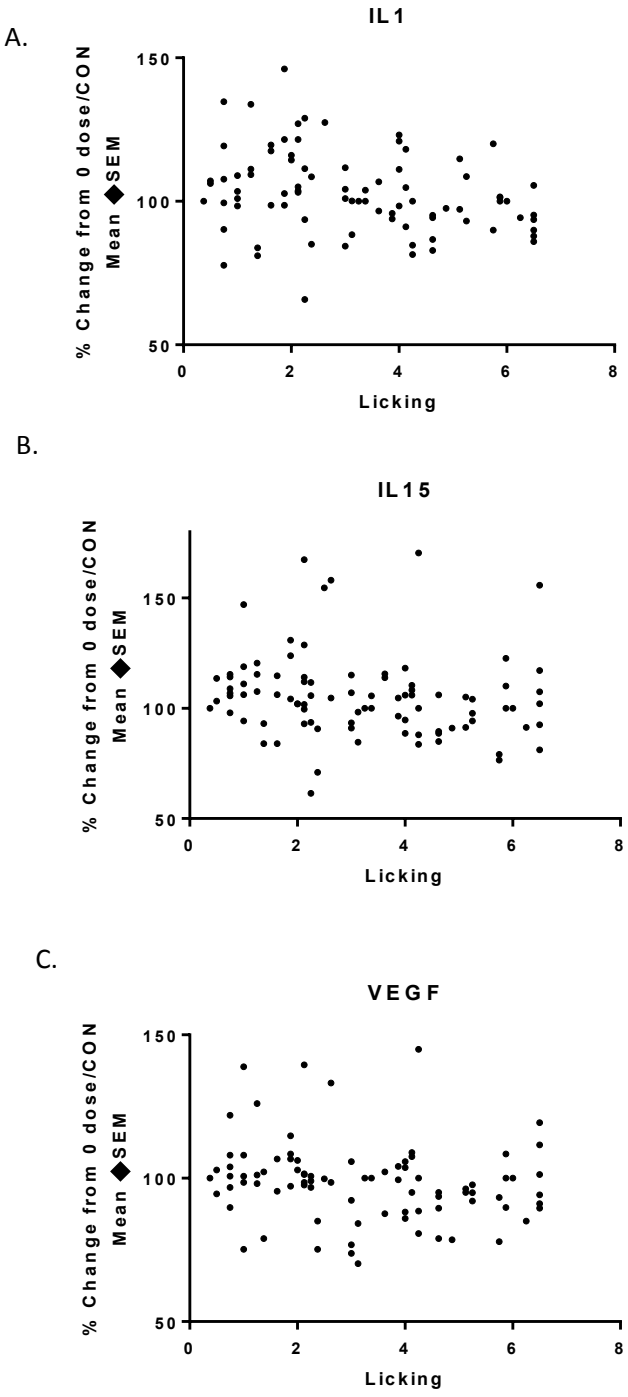



Figure 2.5. Significant negative correlations between maternal licking and A. of IL-1α (r= -.257, p<.02) B. IL-15 (r= -.243, p<.03) and C. VEGF (r= -.249, p<.02).

Table 2.2 Cytokine levels (percent change from control diet/0 BPA) in the mPFC of P10 pups

| Diet | | Control | | High fat | | | ANOVA |
|---|---|---|---|---|---|---|---|
| BPA (µg/kg/day) | | 40 | 400 | 0 | 40 | 400 | |
| **TNFα** | M | 13.4 ± 5.9 | 10.7 ± 9.8 | -2.15 ± 2.0 | -4.2 ± 5.7 | 12.0 ± 8.1 | Sex p<.01 |
| | F | 5.0 ± 6.7 | 7.3 ± 5.9 | 3.8 ± 3.8 | -2.6 ± 3.8 | 4.6 ± 5.9 | Sex x BPA p=.02 |
| **TGFβ** | M | 1.6 ± 5.2 | 8.0 ± 9.7 | 9.3 ± 8.4 | -9.9 ± 9.1 | 1.6 ± 7.9 | Sex p=.05 |
| | F | 0.9 ± 5.9 | 4.1 ± 6.3 | 1.9 ± 6.9 | -4.5 ± 4.2 | 2.2 ± 8.7 | |
| **MCP-1** | M | 4.6 ± 5.8 | 3.3 ± 5.5 | 0.4 ± 3.6 | -1.2 ± 4.1 | 13.2 ± 7.5 | Sex p<.001 |
| | F | 0.2 ± 5.7 | 6.9 ± 4.2 | 8.9 ± 7.6 | 7.4 ± 4.2 | -0.3 ± 3.9 | Sex x BPA p<.01 |
| **IL-1α** | M | 11.1 ± 3.9 | 1.2 ± 6.1 | 4.8 ± 3.4 | -3.9 ± 3.4 | 14.1 ± 5.3 | Sex p<.03 |
| | F | 6.3 ± 4.3 | 2.1 ± 5.3 | -.02 ± 7.3 | -0.9 ± 3.3 | 0.5 ± 4.2 | |
| **IL-1β** | M | 8.7 ± 8.9 | 11.8 ± 8.7 | -7.9 ± 3.8 | -2.5 ± 3.3 | 13.9 ± 10.0 | |
| | F | 12.0 ± 10.0 | 14.3 ± 8.8 | 5.1 ± 7.9 | 1.0 ± 4.5 | 7.7 ± 6.8 | |
| **IL-6** | M | 12.4 ± 7.5 | 20.8 ± 9.1 | 0.6 ± 7.4 | -3.9 ± 2.4 | 15.7 ± 12.3 | Sex p<.001 |
| | F | 13.9 ± 8.4 | 18.6 ± 7.8 | 5.3 ± 11.5 | 3.5 ± 5.7 | 8.0 ± 8.4 | Diet p<.001 |
| **IL-15** | M | 10.5 ± 7.2 | 6.2 ± 8.9 | -1.9 ± 3.4 | 1.2 ± 8.3 | 15.7 ± 9.4 | Sex p<.05 |
| | F | 18.0 ± 5.5 | 5.8 ± 6.1 | -7.1 ± 7.7 | -0.8 ± 4.1 | 3.5 ± 4.3 | |
| **VEGF** | M | 10.3 ± 4.9 | 6.2 ± 7.8 | -0.6 ± 2.2 | -5.7 ± 5.1 | 16.9 ± 6.5 | Sex p<.001 |
| | F | 6.9 ± 2.9 | -2.3 ± 7.9 | -4.6 ± 6.7 | -4.7 ± 5.0 | -.08 ± 2.9 | Sex x BPA p<.01 |

Values are expressed as the mean percent change from control (0 µg/kg/day BPA/control diet) ± SEM.

## Discussion

No interactions between BPA and HFD exposure were observed in maternal behavior or the level of cytokines in the mPFC of P10 pups. However, there were separate effects of HFD and BPA exposure on maternal behavior and inflammation.

The dams fed HFD spent more time nursing pups and less time away from the nest. These effects of HFD on maternal care are supported by previous work showing an increase in nursing and decrease in resting (away from nest) with HFD ingestion (Purcell et al., 2011, but see also Connor et al., 2012). Thus HFD during the metabolically demanding times of pregnancy and postnatal nursing can facilitate maternal behavior. BPA exposure at the higher

dose, on the other hand, resulted in a near significant decrease in the amount of licking behavior exhibited by the dam. This mirrors a pattern in preliminary data from our laboratory in which 400ug/kg/day BPA resulted in less licking than 40ug/kg/day (Sadowski et al., 2011). During the maternal observations, BPA was being administered directly to the pups, not the dam. So the decrease in licking by the dams may be due to BPA-induced changes in the pups, perhaps by alterations in behavior or olfactory cues. Increased maternal licking of pups has been linked to multiple behavioral and cellular alterations in the offspring, including a decrease in behavioral stress response (Liu et al., 1997). This is supported with our data showing the negative correlation between maternal licking behavior and a reduction in inflammatory cytokines, IL-1α, IL-15 and VEGF. Therefore, the reduction in licking with BPA exposure in our rats should result in adults that are more prone to stress as has been reported in the literature (Caldji et al., 1998).

There was evidence in the present study that male offspring, but not females, had increased levels of pro-inflammatory cytokines, TNFα, MCP-1 and VEGF in the mPFC at the highest dose of BPA. The increased presence of cytokines in an early developing brain indicates inflammation due to BPA, which may also shape the behavioral outcomes of the offspring. Previous research has found an increase in anxiety-like behavior and an increase in IL-6 and TNFα following perinatal BPA exposure (Luo et al., 2014). In humans, gestational and neonatal exposure to inflammation results in a higher likelihood of developmental disorders, anxiety and depression, and cognitive impairments to memory, learning, and attention (Bilbo & Schwarz, 2009). The following chapter (3) pursues the possibility of longer-term effects by examining periadolescent social play, anxiety and social recognition behavior of littermates to pups in the current chapter.

Interestingly, perinatal exposure to HFD resulted in a decrease in the protein level of IL-6 in the mPFC, which is contrary to what was reported in the hippocampus of young animals (Bilbo & Tsang, 2010). However, our study was focused on the mPFC, and the impact of perinatal HFD may be region-dependent. The current study adds to the evidence that both HFD and BPA have effects on maternal behavior and cortical inflammation during the early postnatal period. However, they do not appear to interact.

# CHAPTER 3: EFFECTS OF PERINATAL BISPHENOL A AND HIGH FAT DIET ON PERIADOLESCENT PLAY, ADULT ANXIETY AND SOCIAL MEMORY

Among the most common behavioral effects of BPA are sexually dimorphic social and anxiety-based behaviors. Perinatal BPA exposure increases social investigation between female rats, but decreases the amount of play behavior between the females and unexposed male rats (Porrini et al., 2005). Also in the Rissman laboratory, Westenholme et al. (2011) found an increase in approach and solicitation of play behavior and nose-to-nose contact during social interactions in both male and female juvenile C57Bl/6 mice following gestational BPA exposure. Female mice were particularly susceptible to behavioral alterations with decreases in self-grooming and increases in side-by-side interactions that were not as apparent in male animals (Westenholme et al., 2011).

In another study of social interaction, prenatal BPA exposure resulted in an increased latency to habituate to a novel ovariectomized female across trials in both male and female C57Bl/6 mice, suggesting an alteration of social memory (Westenholme et al., 2013). Social memory is also a sexually dimorphic behavior in animal models, with males spending more time investigating than females and females having a longer retention of social memory (Markham & Juraska, 2007). These changes in sex differences in social behaviors are implicating BPA as an endocrine disrupting chemical that is able to alter neural substrates that underlie social behavior.

Further, BPA has been found to abolish or reverse the sex difference usually found in anxiety tasks. Control male rats tend to exhibit more anxiety-like behaviors than the females by spending more time in the closed arms and making fewer crossings into the open arms of the maze (Imhof et al., 1993). Prenatal BPA exposure increases the number of entries and time into the open arms in male rats, suggesting a decrease in anxiety. However, female offspring show a

decrease in the total number of entries into the arms suggesting a decrease in activity (Farabollini et al., 1999), again suggesting that BPA can abolish or reverse sex differences in behavior.

The long-term effects of maternal HFD on offspring juvenile and adult behavior have not been extensively studied, and this includes periadolescent play behavior and adult social recognition. There are some indications that gestational exposure to HFD may increase anxiety in adult male offspring. Male offspring exposed to gestational HFD have been shown to decrease the amount of time spent on the open arms of an EPM (Aslani et al., 2015), suggesting an increase in anxiety. Unfortunately, females were not used in the Aslani et al. (2015) study so a comparison of sex is not possible. Both male and female offspring exposed to gestational HFD were assessed in another EPM study and only males were found to have higher levels of anxiety based on maternal HFD (Bilbo & Tsang, 2010). The current study assessed the effects of combined BPA and HFD exposure on social and anxiety behaviors using periadolescent play observations, social recognition and EPM in adults.

## Methods

*Breeding*

Male and female Long Evans hooded rats (Harlan Laboratories (now Envigo), Indianapolis, IN, USA) were housed in our vivarium for at least one week before being paired for breeding. They were kept on a 12:12 light/dark cycle and were allowed food and water ad libitum. Precautions were taken to reduce the environmental exposure to endocrine disruptors. All animals were housed in BPA-free polysulfone cages, watered with reverse osmosis filtered water in glass bottles, fed a low phytoestrogen food (Harlan 2020X; Teklad Diets, Madison, WI, USA) and exposed to a specialize diet during gestation and through postnatal day (P) 10.

Breeding pairs were placed in suspended wire bottom cages and checked daily for the presence of sperm plugs. The day a sperm plug was detected was recorded as gestational day (G) 0 and the dams were singly housed in a polysulfone shoebox cage. The breeding animals were paired for a maximum of six nights. If no sperm plug was detected after six nights, the male was removed and another male introduced. From the first day of pregnancy, each female was assigned to one of six groups. The N for the control diet and BPA exposures are: 0 µg BPA/kg = 11; 40 µg BPA/kg = 12; 400 µg BPA/kg = 12. The N for the high-fat diet and BPA exposures are: 0 µg BPA/kg = 10; 40 µg BPA/kg = 10; 400 µg BPA/kg = 10.

*BPA Dosing*

BPA was suspended in tocopherol-stripped corn oil at 0, 0.1 mg BPA/ml, or 1.0 mg BPA/ml in order to administer 0 (control), 40 µg BPA/kg , or 400 µg BPA/kg respectively. To dose the adult dams, the required amount (0.4 µl/g body weight) was pipetted onto ½ of a cookie (Newman's Own organic alphabet cookie, vanilla flavor) and given to the animals. The animals readily consumed the cookie making this route of exposure non-stressful and similar to human ingestion of BPA. On G 0 and G 1, the dams were given ½ of a cookie with 0.4 µg/g tocopherol-stripped corn oil. Starting on G 2 through parturition, the dams were given the cookie with the assigned BPA dose. The day of birth was recorded as P 0 and the litters were not disturbed. Then daily from P 1-10, each pup was individually dosed via pipetting the assigned solution (same as dam) directly into its mouth because lactational transfer of BPA is very low (Doerge et al., 2010). Pups were weaned into dyads or triads on P 25.

*Diet*

Starting on G 0 through P 10, the dams were fed either a control diet (CON; 15.8% kcal fat; D10012G, Research Diets Inc., New Jersey, USA) or a high-fat diet (HFD; 45% kcal fat; D12451, Research Diets Inc., New Jersey, USA). The specialized diets were isocaloric for protein. The animals were allowed to feed ad libitum. After P 10, the dams and pups were returned to the standard rat chow (Harlan 2020X).

*Periadolescent Play Behavior*

Two same sex, same treatment animals that were not cagemates were paired to assess play behavior between the ages of P26-40. The animals were isolated in individual cages for one hour before each play session. The animals then were paired for play assessments in a neutral cage four days in a row one hour before the colony lights turned off. The animals' behavior was observed for 20 minutes with one minute time point sampling to classify the behaviors: sniffing, wrestling, chasing, passive contact (no play, but touching), and solitary (no play and not touching). After data collection, the animals were returned to their home cage. Once the animals reached 90 days of age, they were tested on elevated plus maze (EPM) or on social recognition.

*Elevated Plus Maze*

At P 90 (adulthood), the rats were tested for anxiety behavior using the EPM. The floor of the EPM apparatus was 50 cm tall with two open arms and two closed arms that were each 50 cm long and 10.5 cm wide. The two closed arms were enclosed with walls 33.5 cm tall. Each animal was placed on the center and allowed to explore the maze for one 5 minute trial. Time spent in each arm, time in the center and number of entries into each arm were recorded. The

operational definition of "entry" was more than half of the animal's body had to enter the arm in order to be counted and timed.

*Adult Social Recognition*

At P90, another group of animals were tested for social memory using the social recognition task using same sex juveniles (P21-30). This task has been previously used in our laboratory (Markham & Juraska, 2007). At least twenty minutes before the task began, each juvenile was marked on its back with a different colored non-toxic marker for easy identification during the task. To test social memory, adult animals were introduced to and allowed to investigate a same-sex juvenile for thirty seconds. After the initial introduction, the juvenile animal was removed and the adult was placed back with its cagemate for a delay period of 15, 45, 90 or 120 minutes. After the delay period, the same juvenile (familiar) and a novel juvenile were placed in a cage with the adult. The time the adult spent investigating each juvenile was recorded for three minutes. In theory, an animal with good social memory will spend more time investigating the novel juvenile rather than the familiar juvenile. There were three days of habituation, which were identical to testing except the delay period was always 30 minutes. Data were collected during habituation but was not included in the data analysis. Following habituation, the delay periods were randomized between animals for the four days of testing. The adult animals never saw the same juveniles on more than one day.

*Statistical Analysis*

To analyze the periadolescent play behavior, the categories of sniffing, chasing and wrestling were combined into one "social behavior" category. More than one animal was tested

per litter, and the average counts of each category: social behavior, passive contact, and solitary for males and females per litter were calculated and used in the analysis. Each behavior was averaged across days 1 - 2 and days 3 - 4. A 3 (BPA treatment) x 2 (diet) x 2 (sex) x 2 (days) repeated measures ANOVA was used a 3 (BPA treatment) x 2 (diet) x 2 (sex) ANOVA was used to analyze the EPM data: time in open arm, time in closed arm, open arm entries, closed arm entries, and time in center. A 3 (BPA treatment) x 2 (diet) x 2 (sex) x 4 (delay) repeated measures ANOVA was used to analyze the percent of time spent investigating the novel juvenile at each delay of the social recognition task. Significant interactions for all measures were further analyzed using a LSD post hoc test.

## Results

*Periadolescent Play Behavior*

There were no main effects of treatment or diet. There was a significant interaction between day and BPA treatment for play, $F(2, 160)=6.34$; $p<.01$ (Fig. 3.1.). No significant differences were found on days 1-2 of play behavior. However, differences emerged on days 3-4. Play behaviors (sniffing, wrestling and chasing) were significantly higher in control animals than in the 400 µg BPA/kg animals ($p<.05$), while the 40 µg BPA/kg animals appear to show a decrease in play, the difference with the control animals was only $p <.10$. There was also a sex difference in the amount of play behavior with males exhibiting more play than females, $F(1,160)=6.70$, $p<.05$.

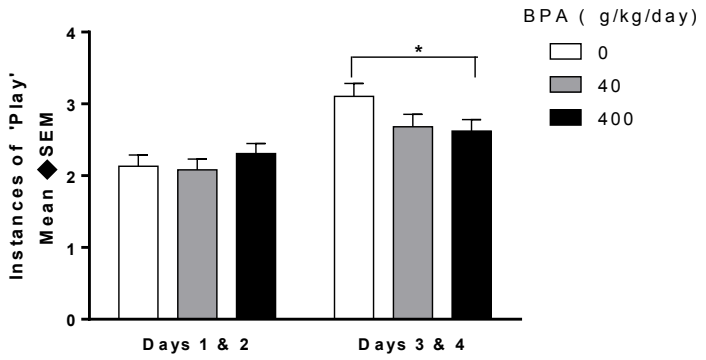



Figure 3.1. Animals exposed to 400 µg BPA/kg/day decreased the amount of play behavior compared to controls on days 3 and 4.

For the passive contact, there was also a significant interaction between days and BPA treatment, $F(2,160)=5.67$, $p<.01$ (Fig. 3.2). Once again, there were no significant differences on days 1-2. However, on days 3-4, the 400 µg BPA/kg animals spent significantly more time in passive contact than the control group ($p<.05$).

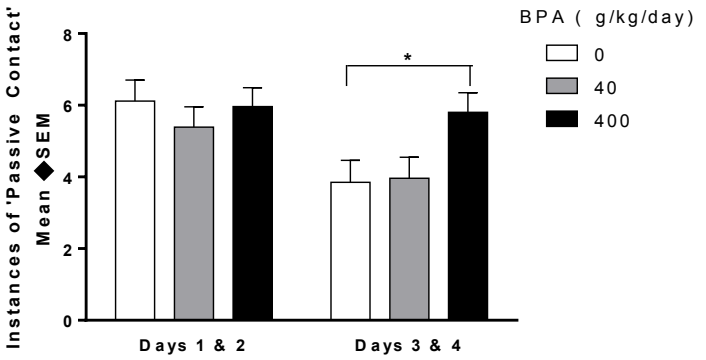



Figure 3.2. Animals exposed to 400 µg BPA/kg/day spent significantly more time engaging in passive contact (touching but no play) than the control animals.

Finally, there was a day x treatment interaction for time alone, $F(1,160)=4.00$, $p=.047$ (Fig. 3.3). Days 1-2 showed no significant differences. Once again, on days 3-4, 40 µg BPA/kg

animals spent more time alone than the control animals ($p<.05$). There was a significant main effect of sex in the amount of time spent alone, $F(1,160)=14.08$, $p<.001$; with females spending more time alone than males. There were no significant differences in behavior based on maternal diet on any measure.

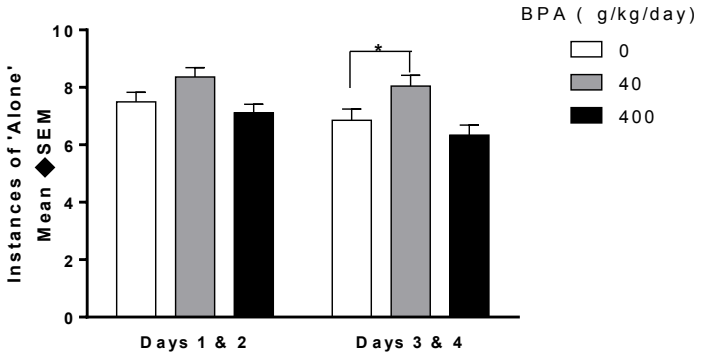



Figure 3.3. Animals perinatally exposed to 40 µg BPA/kg/day spent significantly more time alone than the control animals on days 3 and 4.

*EPM*

There were no significant differences due to BPA or diet in the EPM for any of the measures (time in open arms, time in closed arms, number of crossings into the open arms, number of crossings into the closed arms). There was a significant sex difference in the number of entries into open arms, with females entering the open arms more often, $F(1,100)=8.52$, $p<.01$. This is a frequently observed sex difference in the EPM (Imhof et al., 1993).

Previous research has shown a reversal of sex differences with BPA exposure (Farabollini et al., 1999). In order to compare our results to previous literature, a further analysis was restricted to the control diet animals, and there was a significant sex by BPA treatment interaction both in the time spent in the closed arms, $F(2,62)=3.84$, $p<.05$, and time spent in the open arms, $F(2,62)=3.69$, $p<.05$. Post hoc analysis found a significant sex difference between males and females in the time spent in the closed arms ($p<.01$) and time spent in the

open arms (p<.01), but 400 µg BPA/kg/day abolished the sex difference. Males showed a significant dose-dependent increase in the time in open arms with BPA treatment, F(2, 35)=3.70, p<.05, suggesting a anxiolytic and female-like effect of BPA. Females did not show significant differences due to BPA treatment.

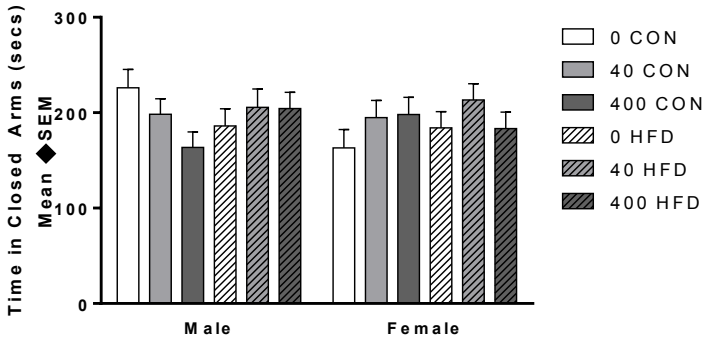



Figure 3.4. Perinatal exposure to BPA and HFD did not change the amount of time spent in the closed arms of the EPM.

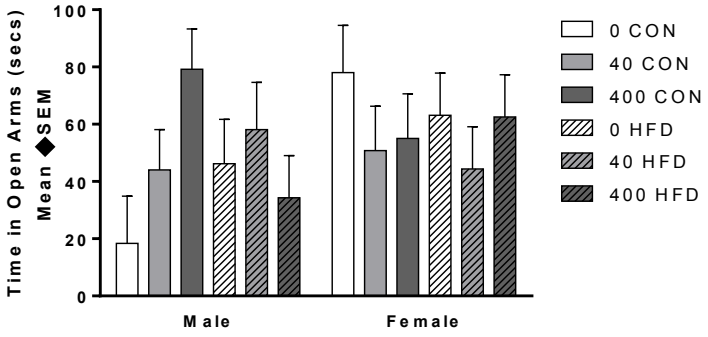



Figure 3.5. No significant differences were found in the amount of time spent in open arms of the EPM based on maternal diet or BPA exposure.

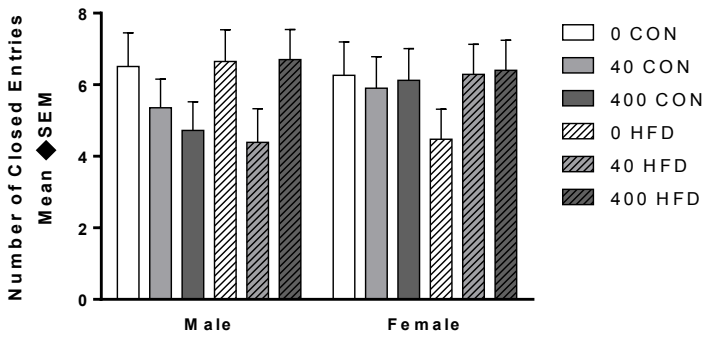



Figure 3.6. There were no significant differences in number of closed arm entries in the EPM due to maternal diet or BPA exposure.

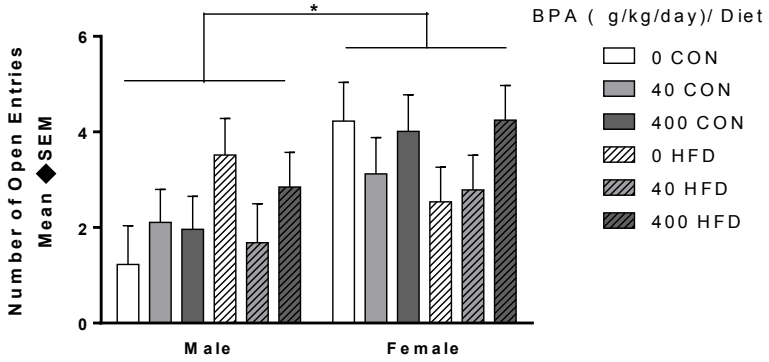



Figure 3.7. There was a significant female- biased sex difference in the number of open arm entries in the EPM. But there were not significant differences due to maternal diet or BPA exposure.

*Social Recognition*

There were no significant differences in social recognition based on either BPA or diet ($p>.05$).

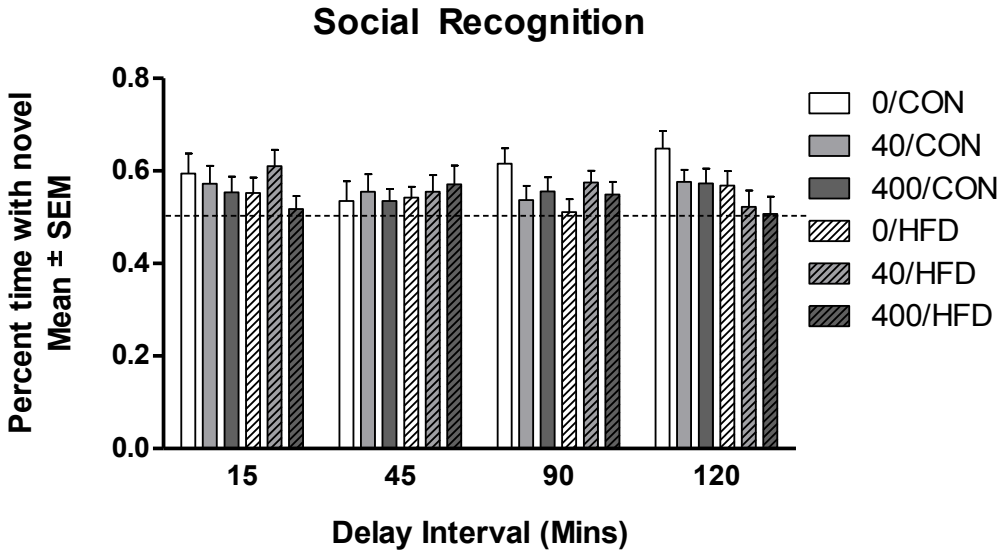



Figure 3.8. The percent of total time spent with the novel animal. There were no significant differences in maternal diet or BPA exposure in the social recognition task.

## Discussion

Perinatal exposure to HFD did not have an effect on any of the behaviors investigated in the current study; however, perinatal BPA exposure had several significant long-term alterations on behavior. Periadolescent play behavior was susceptible to BPA exposure with changes in behavior evident on days 3-4 of observation. Animals in the control group spent more time engaged in play behaviors, while animals exposed to 40 µg BPA/kg were alone most often, and animals exposed to 400 µg BPA/kg were more often in passive contact. BPA also affected the elevated plus maze in animals from the control diet group, with males perinatally exposed to BPA spending more time on the open arms, suggesting an anxiolytic effect. Social recognition was not affected by BPA exposure.

Our results of periadolescent play behavior agree with previous studies that found a decrease in play (Porrini et al., 2005). However, in our data, BPA did not produce sex-specific

effects as had been previously found. Although sex differences were found with males spending more time playing and females spending more time alone, there were not sex-specific changes due to BPA in these measures.

Along with the striatum and medial amygdala, play behavior is mediated by the mPFC, with inactivation of either the prelimbic or infralimbic subregions of the mPFC significantly reducing play (van Kerkhof et al., 2013; Vanderschuren et al., 2016). Because play behavior was assessed weeks after BPA exposure ended, these data suggest that gestational BPA exposure may alter the longer-term structure of brain regions including the mPFC. This will be investigated by quantifying the numbers of neurons, glia, synapses and microglia in the mPFC of these animals in Chapter 5.

Anxiety behavior tested via the EPM was not affected by HFD, and there was no an interaction between BPA and HFD. Evidence of an anxiolytic effect in males due to BPA exposure was uncovered when the animals in the control diet group were analyzed separately, which supports previous literature. Further, the loss of a sex difference in the EPM or even a reversal of a sex difference has been found in BPA toxicology studies (Farabollini et al., 1999).We also find this effect of perinatal BPA exposure to 400 μg /kg/day where the anxiolytic effect on males abolished the sex difference. Control male animals spend more time in the closed arms, but the 400 μg /kg/day BPA exposed males increased their time on the open arms to female levels.

Anxiety behavior is influenced by the mPFC through cortical-amygdalar circuitry (Tovote, et al., 2015). Alterations in the anatomy of the mPFC by BPA could also affect anxiety behavior displayed in the EPM. For example, both excitotoxic lesions and infusions of

muscimol into the mPFC, which inactivate GABAa receptors, reduce anxious behavior in the EPM (Shah & Treit, 2003; Shah et al., 2004), as was observed in the current study in male animals exposed to BPA. This is a level of influence that would need to be pursued beyond this dissertation as it is beyond the scope of the current study to investigate this mechanism.

Surprisingly, there was no effect of gestational BPA or HFD exposure on social recognition behavior. Social recognition is also dependent, in part, upon the PFC (Loiseau et al., 2008), along with the striatum, and amygdala (van Kerkhof et al., 2013; Vanderschuen et al., 2016) while social recognition depends upon connections between the prefrontal cortex, hypothalamus and lateral septum (Albers, 2012). Exposure to the EPM activates circuitry between the prefrontal cortex, amygdala and the bed nucleus of stria terminals (Adhikari, 2014). The differing results suggest that not all prefrontal cortex circuits are susceptible to perinatal BPA or HFD exposure. However, caution is needed in interpreting the social recognition results since the expected effects of time delays on memory were not observed. In fact, the majority of the animals preferred to investigate the novel animal rather than the familiar at all the delays, unlike previously reported results (Markham & Juraska, 2007).

The data from the current study suggest that BPA exposure, but not HFD, affects some social and anxiety behaviors. In order to further define the effects of BPA on brain, the number of neurons, glia, synaptophysin and microglia in the mPFC will be examined in chapter 5.

# CHAPTER 4: EFFECTS OF PERINATAL BISPHENOL A AND HIGH FAT DIET ON GENE EXPRESSION IN THE PREFRONTAL CORTEX AT P10 AND P90

Previous chapters have outlined the effects of perinatal BPA and HFD on maternal behavior, postnatal inflammation, periadolescent play behavior and adult behaviors. HFD altered maternal behavior, BPA exposure altered periadolescent play behavior and performance on the EPM, while both BPA and HFD were found to alter postnatal pro-inflammatory markers in the mPFC. To further assess the underlying molecular mechanisms of the behavioral changes the current chapter investigates the expression of genes related to hormone receptors (estrogen receptor (*Er)α, Erβ*, estrogen related receptor *(Err)γ*, androgen receptor *(Ar*), steroidogenic acute regulatory protein (*StAr),* cholesterol side-chain cleavage enzyme *(Cyp11a1)*), inflammatory or oxidative stress markers (allograft inflammatory factor 1 (*Aif1),* complement factor *(C4a), C4b,* catalase *(Cat*), superoxide dismutase *(Sod) 1, Sod2,* glutaredoxin *(Glrx))* and markers of neuroanatomical structural change (caspase (*Casp3*)) in the cortex at P10 and adulthood.

Both perinatal BPA and HFD can change gene expression. Perinatal BPA exposure has been found to affect gene expression of *Erα, Erβ, Errγ*, as well as dopamine and serotonin receptor-related proteins in the prefrontal cortex of young rats at P21 and mice at P 28 (Kundakovic et al., 2013; Castro et al., 2015). In addition, the differences in gene expression of *Erα, Erβ,* and *Errγ* following prenatal BPA exposure have been found to be due to changes in DNA methylation at P28 (Kundakovic et al., 2013). Gestational HFD through P40 is known to affect oxytocin-related proteins in the mPFC at P40 (Hehar et al., 2016). Maternal HFD through gestation and lactation also alters opioid and dopamine-related proteins in the mPFC of adult male mice, indicating effects in gene expression (Vucetic et al., 2016).

In the current study, we examined the level of expression of genes from the mPFC of littermates at P10 and P90, allowing for a direct comparison of the short and long-term impact of BPA and HFD.

## Methods

*RNA Isolation and One-Step Real Time qPCR*

On P10 and P90, one male and one female from each litter was euthanized via CO2 and the brain was immediately removed, snap frozen in liquid nitrogen, and stored in a -80C degree freezer. The mPFC tissue was collected from frozen tissue and transferred to Y-X Pan's laboratory where Diego Hernandez-Saavedra conducted the RNA isolation and qPCR analyses. Genes related to hormones and hormone receptors (*Erα, Erβ, Errγ, Ar, StAr, Cyp11a1*), inflammation or oxidative stress (*Aif1, C4a, C4b, Cat, Sod1, Sod2, Glrx)* and apoptosis (*Casp3*) were chosen for investigation. All tissue was coded to conceal treatment group. Total RNA was isolated using TRI reagent (Sigma, St. Louis, MO, USA), followed by Direct-zol™ RNA MiniPrep according to manufacturer's instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real time PCR was performed using the StepOnePlus™ Real-Time PCR System with Power SYBR® Green PCR Master Mix using the respective forward and reverse primer for each gene (Table 4.1), and were designed by Vector NTI software (Invitrogen Corporation) and synthesized by Integrated DNA Technologies (www.idtdna.com). Standard curves with a slope of -3.30 (SEM 0.20) and R2≥ 0.99 were accepted. A housekeeping gene whose expression was not affected by treatment was used to normalize the gene expression data: 60S ribosomal protein (RpL7a).

**Table 4.1.** Primers used for qPCR for cortical samples.

| Gene (Ensembl /No.Transcript ID) | Position | Sequence |
|---|---|---|
| ***Er-α*** (ENSRNOT00000081017) | +2 F | CACACACGCTCTGCCTTGA |
| | +68 R | GACGGAAGGAAGGAATGTGC |
| ***Er-β*** (ENSRNOT00000042682) | +754 F | ATCCAGGAGAGAACGGTGTGGGT |
| | +825 R | AGGCAGTGTACCTGCTCGCTAGAAC |
| ***Err-γ*** (ENSRNOT00000003489) | +197 F | GCGCACATGGATTCGGTAGAA |
| | +268 R | GCAGAGAAGCTCTTCTTCGTAGTGC |
| ***Ar*** (ENSRNOT00000009129) | +2508 F | GAAATGGGACCTTGGATGGAGA |
| | +2585 R | TAAAACGTGGTCCCTGGTACTGTC |
| ***Star*** (ENSRNOT00000020606) | +686 F | CTTTGGGGAGATGCCTGAGC |
| | +765 R | CAGCCAGTGGATGAAGCACC |
| ***Cyp11a1*** (ENSRNOT00000010831) | +563 F | TTACACAGACGCATCAAGCAGC |
| | +638 R | AGGCAAAGCGGAATAGGTCATC |
| ***Cat*** (ENSRNOE00000078559) | +220 F | TTGACAGAGAGCGGATTCCTGAGA |
| | +297 R | CGTGGGTGACCTCAAAGTATCCAA |
| ***Sod1*** (ENSRNOT00000002885) | +239 F | CAGCGGATGAAGAGAGGCA |
| | +310 R | ACACATTGGCCACACCGTC |
| ***Sod2*** (ENSRNOT00000025794) | +738 F | GTTTGCAAGAAGTGAAGC |
| | +801 R | ACTACAAAACACCCACCA |
| ***Glrx*** (ENSRNOT00000016372) | +262 F | CGGAGCAAGAACAGTTCCTCGG |
| | +331 R | GGAGAGTAGATCACTGCATCCGCC |
| ***Aif1*** (ENSRNOT00000001135) | +144 F | CTGAAAGCCCAACAGGAAGAGA |
| | +207 R | ACTTGGGATCATCGAGGAAGTG |
| ***C4a*** (ENSRNOT00000086027) | +902 F | AGCAGGGGAAGAAGTCTT |
| | +979 R | AGATGGAGATGTGAGTCTGG |
| ***C4b*** (ENSRNOT00000031704) | +517 F | ACGGTCACAGTAGAGAACTC |
| | + 592 R | CTGTGAAGATGGATGTGG |
| ***Casp3*** (ENSRNOT00000014095) | +506 F | TCTGACTGGAAAGCCGAAACTCTTC |
| | +588 R | TTCCACTGTCTGTCTCAATACCGCA |

*Statistical Analysis*

A 3 (treatment) x 2 (diet) x 2 (sex) ANOVA was used to analyze each gene from the mPFC sample. LSD posthoc tests were used to compare each dose to control for any interacting factors.

## Results

*Gene Expression in the P10 mPFC*

The results of the qPCR analyses on the P10 brains are in Tables 4.2. Several of the analyzed genes exhibited a sex difference in expression levels in the PFC with females always

showing higher expression: *Erα* ($p<.05$), *Sod1* ($p<.04$), *Aif1* ($p<.02$), *C4a* ($p<.001$), and *Casp3* ($p<.005$). *Erα,* $F(2,89)=7.39$, $p<.002$, and *Sod1*, $F(2,89)=3.75$, $p<.03$, both showed significant increases of expression levels in the PFC due to BPA treatment in both sexes. Posthoc comparisons found animals exposed to 40 µg BPA/kg ($p<.02$) and 400 µg BPA/kg ($p<.001$) had higher expression levels of *Erα* in the PFC than control animals (Fig. 4.1A); likewise, *Sod1* expression was significantly higher following both 40 and 400 µg BPA/kg doses compared to controls ($p<.02$). HFD caused a significant decrease in gene expression of *Sod2* $F(1,89)=6.85, p<.02$.

The only significant interactions between BPA treatment and diet in gene expression were found in *Errγ*, $F(2,89)=4.47, p<.02$ (Fig. 4.1B) and *Glrx*, $F(2,89)=3.46, p<.04$ (Fig. 4.1.C). Post hoc analysis showed *Errγ* to be significantly higher with HFD when there was no exposure to BPA ($p<.001$), but the level of *Errγ* in animals exposed to 40 µg/kg/day BPA was lower than controls only in the HFD group ($p<.05$). *Glrx* was significantly higher at 400 µg/kg/day BPA exposure only in the animals given HFD ($p<.05$). The remainder of the genes investigated did not show significant differences: *Erβ, Ar, Star, Cyp11a1, Cat, and C4b*. In order to assess the possible associations between maternal behavior and resulting gene expression of the offspring, a two-tailed Pearson's correlation was conducted comparing each maternal behavior to each gene. None of the behaviors or gene expression levels were significantly correlated (data not shown).

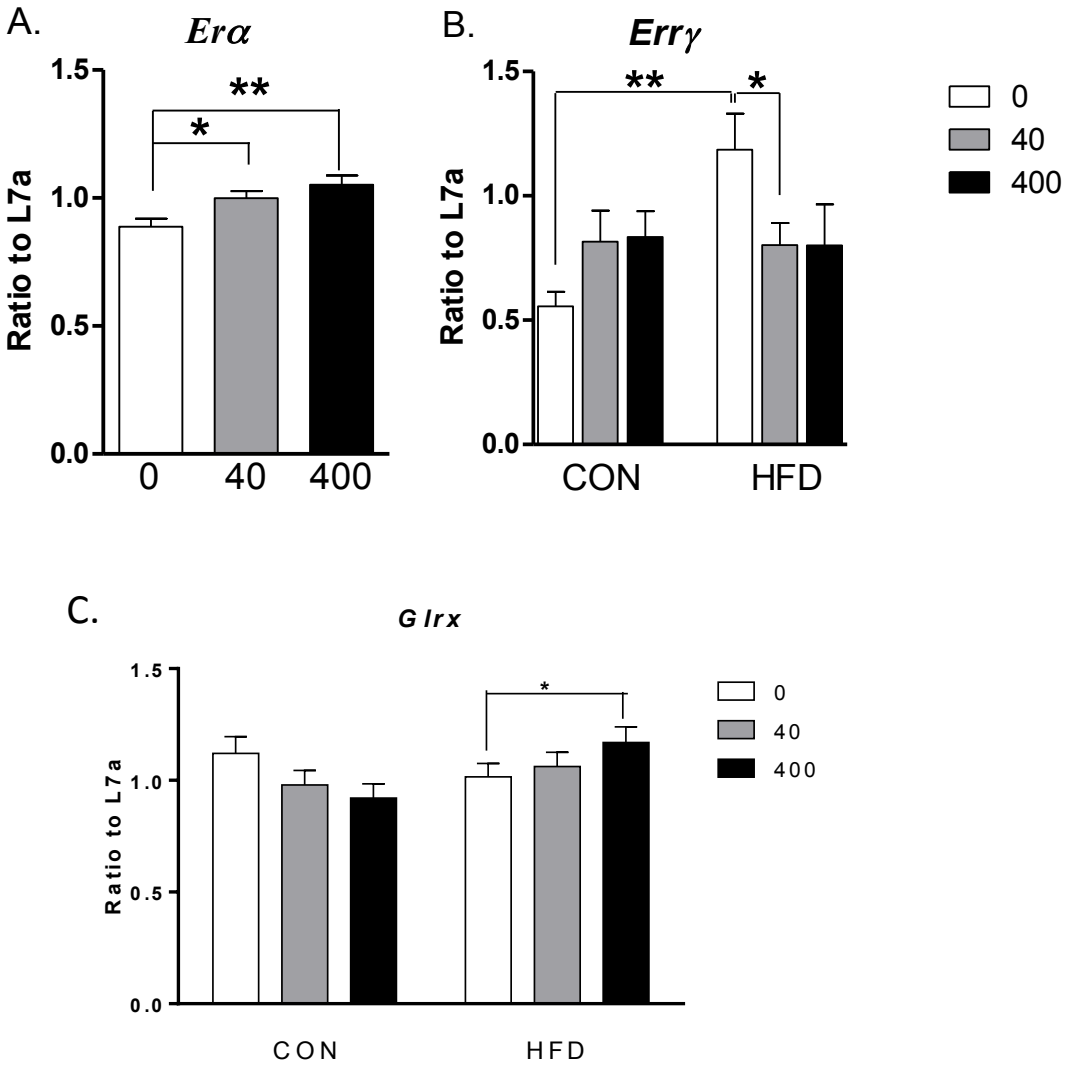



Figure 4.1. Gene expression of receptors associated with estrogen in the mPFC. A) *Erα* showed a significant increase in gene expression in the PFC of P10 pups with perinatal exposure to BPA. B) *Errγ* presented with a significant interaction in PFC gene expression between BPA treatment and diet in the P10 animals. *Errγ* was increased with HFD, and then decreased with 40 µg/kg/day BPA exposure. C) *Glrx* was significantly higher at 400 µg/kg/day BPA exposure only in the animals given HFD ($p<.05$)*$p<.05$; **$p<001$.

| Table 4.2 Gene expression from the mPFC of P10 male & female rats | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Diet** | | **Control** | | | **High Fat Diet** | | | **ANOVA** |
| **BPA (µg/kg/day)** | | **0** | **40** | **400** | **0** | **40** | **400** | |
| **Hormone receptors and enzymes** | | | | | | | | |
| Erα | M | 0.74 ± 0.06 | 0.99 ± 0.02 | 0.97 ± 0.06 | 0.85 ± 0.04 | 1.01 ± 0.06 | 1.08 ± 0.04 | Sex p< .05 |
| | F | 0.98 ± 0.04 | 0.99 ± 0.06 | 1.05 ± 0.04 | 0.96 ± 0.06 | 0.94 ± 0.04 | 1.13 ± 0.11 | BPA p< .002 |
| Erβ | M | 0.88 ± 0.09 | 0.93 ± 0.10 | 0.98 ± 0.11 | 1.20 ± 0.10 | 0.80 ± 0.10 | 0.79 ± 0.08 | |
| | F | 0.81 ± 0.09 | 0.75 ± 0.06 | 0.75 ± 0.06 | 0.89 ± 0.12 | 0.95 ± 0.11 | 0.88 ± 0.10 | |
| Errγ | M | 0.59 ± 0.09 | 0.91 ± 0.18 | 0.91 ± 0.17 | 1.24 ± 0.24 | 0.78 ± 0.14 | 0.79 ± 0.21 | Diet*BPA p<.02 |
| | F | 0.52 ± 0.06 | 0.64 ± 0.08 | 0.75 ± 0.10 | 1.08 ± 0.15 | 0.87 ± 0.11 | 0.82 ± 0.16 | |
| Ar | M | 1.02 ± 0.10 | 0.95 ± 0.07 | 0.89 ± 0.09 | 0.84 ± 0.09 | 0.88 ± 0.09 | 0.95 ± 0.10 | |
| | F | 0.85 ± 0.09 | 0.76 ± 0.05 | 0.80 ± 0.08 | 0.85 ± 0.09 | 0.83 ± 0.06 | 0.88 ± 0.10 | |
| Star | M | 1.32 ± 0.13 | 1.06 ± 0.09 | 1.13 ± 0.07 | 1.21 ± 0.09 | 1.20 ± 0.08 | 1.15 ± 0.08 | |
| | F | 1.46 ± 0.09 | 1.17 ± 0.08 | 1.20 ± 0.11 | 1.22 ± 0.11 | 1.13 ± 0.08 | 1.19 ± 0.11 | |
| Cyp11a1 | M | 0.91 ± 0.12 | 0.65 ± 0.10 | 1.10 ± 0.21 | 1.14 ± 0.21 | 1.29 ± 0.23 | 1.18 ± 0.28 | |
| | F | 0.79 ± 0.26 | 1.37 ± 0.20 | 1.24 ± 0.25 | 1.24 ± 0.15 | 1.30 ± 0.22 | 0.89 ± 0.03 | |
| **Inflammation or Oxidative Stress Markers** | | | | | | | | |
| Cat | M | 0.96 ± 0.02 | 1.03 ± 0.02 | 0.98 ± 0.02 | 0.96 ± 0.04 | 0.97 ± 0.04 | 0.94 ± 0.01 | |
| | F | 1.03 ± 0.04 | 1.00 ± 0.02 | 1.04 ± 0.03 | 0.98 ± 0.02 | 0.98 ± 0.01 | 1.04 ± 0.06 | |
| Sod1 | M | 0.86 ± 0.06 | 0.99 ± 0.03 | 0.96 ± 0.02 | 0.91 ± 0.03 | 1.01 ± 0.04 | 1.03 ± 0.03 | Sex p<.04 |
| | F | 1.00 ± 0.01 | 1.04 ± 0.04 | 0.97 ± 0.05 | 0.96 ± 0.02 | 1.03 ± 0.03 | 1.07 ± 0.04 | BPA p<.03 |
| Sod2 | M | 1.15 ± 0.01 | 1.18 ± 0.09 | 1.05 ± 0.05 | 1.02 ± 0.04 | 1.08 ± 0.08 | 1.04 ± 0.08 | Diet p<.02 |
| | F | 1.18 ± 0.08 | 1.25 ± 0.11 | 1.04 ± 0.08 | 0.97 ± 0.08 | 0.93 ± 0.11 | 0.94 ± 0.15 | |
| Glrx | M | 1.06 ± 0.03 | 1.01 ± 0.03 | 1.00 ± 0.01 | 0.95 ± 0.05 | 1.02 ± 0.04 | 1.05 ± 0.07 | Diet x BPA p<.04 |
| | F | 1.18 ± 0.06 | 0.93 ± 0.16 | 0.83 ± 0.15 | 1.06 ± 0.05 | 1.10 ± 0.04 | 1.24 ± 0.12 | |
| Aif1 | M | 1.12 ± 0.13 | 1.08 ± 0.08 | 1.12 ± 0.06 | 1.09 ± 0.10 | 1.24 ± 0.06 | 1.13 ± 0.08 | Sex p<.02 |
| | F | 1.26 ± 0.11 | 1.23 ± 0.09 | 1.31 ± 0.10 | 1.39 ± 0.10 | 1.17 ± 0.14 | 1.44 ± 0.11 | |
| **Complement Pathway** | | | | | | | | |
| C4a | M | 0.99 ± 0.11 | 0.78 ± 0.04 | 0.83 ± 0.08 | 0.82 ± 0.05 | 0.89 ± 0.04 | 0.93 ± 0.07 | Sex p<.001 |
| | F | 1.00 ± 0.06 | 0.93 ± 0.05 | 1.13 ± 0.14 | 1.18 ± 0.11 | 0.97 ± 0.05 | 1.29 ± 0.09 | |
| C4b | M | 1.34 ± 0.14 | 0.79 ± 0.08 | 0.94 ± 0.15 | 1.17 ± 0.13 | 1.13 ± 0.11 | 1.11 ± 0.26 | |
| | F | 0.95 ± 0.18 | 1.15 ± 0.14 | 1.19 ± 0.12 | 1.24 ± 0.19 | 1.08 ± 0.13 | 1.46 ± 0.24 | |
| **Apoptosis** | | | | | | | | |
| Casp3 | M | 0.94 ± 0.03 | 0.99 ± 0.03 | 0.88 ± 0.03 | 0.90 ± 0.04 | 0.89 ± 0.07 | 0.99 ± 0.05 | Sex p<.005 |
| | F | 1.00 ± 0.05 | 1.12 ± 0.04 | 1.03 ± 0.07 | 0.96 ± 0.05 | 1.00 ± 0.03 | 1.14 ± 0.13 | Diet x BPA p=.056 |

*Gene Expression in the P90 mPFC*

The results of the gene expression analysis on the cortex at P90 are in Table 4.3. Significant sex differences were found in several of the analyzed genes: *Sod2* (p<.001), *Aif1*

(p<.04), *C4a* (p<.01), *C4b* (p<.05), *Casp3* (p=.05). Females had higher levels of gene expression of *Sod2, Aif1* and *Casp3*; whereas males had higher levels of gene expression in *C4a* and *C4b.* HFD caused a long-term increase in the expression of genes related to hormone receptors, oxidative stress and apoptosis, including *Erα,* $F(1,66)=4.63$; $p<.05$, *Errγ*,$F(1,66)=4.37$; $p<.05$, *Cat*,$F(1,66)=8.92$; $p<.005$, and *Casp3*, $F(1,65)=4.74$; $p<.05$. There were no main effects of BPA on any of the analyzed genes.

However, BPA interacted with HFD to cause a long-term effect in several genes, *Ar,* $F(2,66)=3.82$; $p<.05$, *Erβ,*$F(2,66)=3.40$; $p<.05$, *Glrx,* $F(2,64)=3.48$; $p<.05$ (Fig. 4.2). Post hocs conducted on *Ar* showed a significant decrease in gene expression following perinatal HFD exposure compared to CON diet ($p<.02$). Further, *Ar* was significantly decreased between 0 and 40 µg/kg/day BPA ($p=.05$) with a trend towards a decrease between 0 and 400 µg/kg/day BPA ($p=.07$). Animals in the HFD groups had a trend towards an increase with 40 µg/kg/day BPA ($p=.06$).

Post hocs conducted on *Erβ* showed that within the CON diet groups, there was a significant reduction in the level of gene expression in animals with 0 BPA exposure and those exposed to 400 µg/kg/day ($p<.02$). However, there were no differences within the groups of animals fed HFD.

Post hoc tests following the interaction between diet and BPA revealed a trend towards a decrease in the level of gene expression of *Glrx* between 0 and 400 µg/kg/day BPA within animals fed a CON diet($p=.057$). However, there were no significant differences between animals fed HFD.

There was a three way interaction of HFD, BPA and sex in *C4a,* $F(2, 65)=3.38$; $p<.04$ (Fig. 4.3). However, post hocs revealed no significant differences between groups. A BPA by sex interaction was found in expression of *Erα,* $F(2,66)=3.61$; $p<.05$, and a post hoc test revealed an increase in the level of gene expression in females following 400 µg/kg/day BPA exposure ($p<.05$), but no differences in males (Fig. 4.4).

HFD by sex interaction was found in the gene expression of *Glrx*, $F(1,64)=4.84$; $p<.05$. Female animals fed a HFD in combination with 400 µg/kg/day BPA exposure had a significant increase in the level of gene expression compared to the 0 BPA group of *Glrx* ($p<.04$). However, males did not have significant change. No HFD or BPA effects or interactions were observed in gene expression of *Aif1, Star, Cyp11a21, Sod1, C4b, Sod2*.

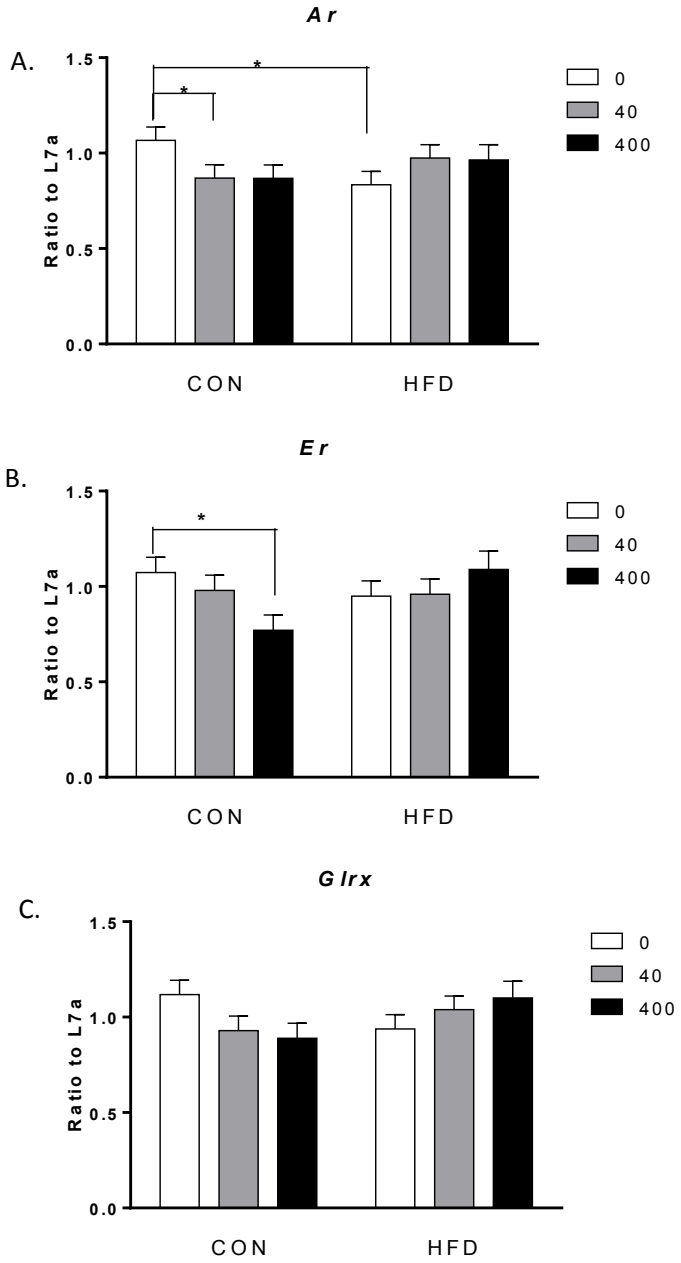



Figure 4.2. Perinatal BPA exposure and HFD interacted to affect the gene expression of *Ar*, *Erβ*, and *Glrx*. A) Levels of gene expression of *Ar* were significantly lower following perinatal HFD exposure compared to CON diet and *Ar* was significantly lower in animals exposed to 40 µg/kg/day BPA compared to controls. B) Animals exposed to 400 µg/kg/day BPA and CON diet had significantly less expression of *Erβ*, whereas there was no significant effects following HFD exposure. C) Posthocs conducted on the levels of gene expression of Glrx following perinatal HFD or BPA exposure found a decrease between 0 and 400 µg/kg/day BPA and CON diet that was approaching significance (p=.057) and no differences in the animals exposed to HFD. *p<.05

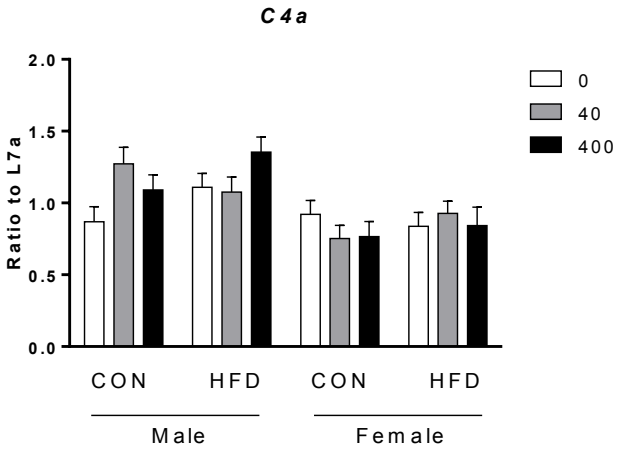



Figure 4.3. There was a significant three way interaction of HFD, BPA and sex in *C4a.* However, post hocs revealed no significant differences from the 0 µg/kg/day control groups.

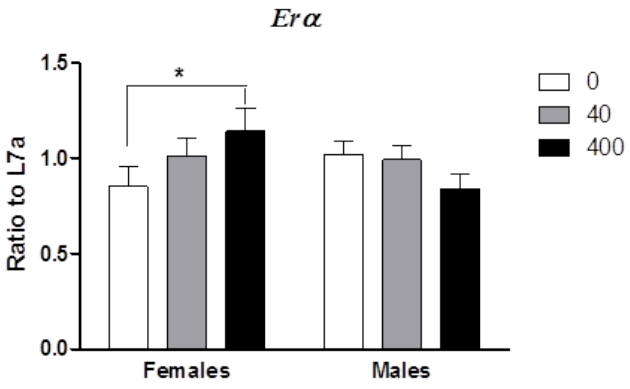



Figure 4.4. Perinatal BPA exposure affected the gene expression level of *Erα* differently in the cortex of adult male and female animals. *Erα* was also significantly increased in P10 animals, suggesting a long lasting increase in the level of expression due to perinatal BPA exposure.

Table 4.3 Gene expression from the cortex of P90 male & female rats

| Diet | | Control | | | High Fat Diet | | | ANOVA |
|---|---|---|---|---|---|---|---|---|
| BPA (µg/kg/day) | | 0 | 40 | 400 | 0 | 40 | 400 | |
| **Hormone receptors and enzymes** | | | | | | | | |
| **Erα** | M | 0.92 ± 0.12 | 1.00 ± 0.06 | 0.81 ± 0.08 | 1.10 ± 0.10 | 0.98 ± 0.08 | 0.88 ± 0.11 | Diet p<.05 |
| | F | 0.84 ± 0.06 | 0.84 ± 0.12 | 1.01 ± 0.19 | 0.86 ± 0.14 | 1.20 ± 0.12 | 1.32 ± 0.08 | BPA x Sex p<.05 |
| **Erβ** | M | 1.10 ± 0.09 | 1.08 ± 0.15 | 0.76 ± 0.11 | 1.03 ± 0.10 | 0.91 ± 0.16 | 0.99 ± 0.09 | Diet x BPA p<.05 |
| | F | 1.05 ± 0.08 | 0.88 ± 0.10 | 0.78 ± 0.07 | 0.88 ± 0.15 | 1.01 ± 0.08 | 1.15 ± 0.08 | |
| **Errγ** | M | 1.19 ± 0.14 | 1.37 ± 0.20 | 1.05 ± 0.15 | 1.36 ± 0.09 | 1.32 ± 0.11 | 1.45 ± 0.08 | Diet p<.05 |
| | F | 1.48 ± 0.15 | 1.05 ± 0.12 | 1.23 ±0.11 | 1.41 ± 0.08 | 1.50 ± 0.13 | 1.29 ± 0.05 | |
| **Ar** | M | 1.00 ± 0.11 | 0.93 ± 0.13 | 0.74 ± 0.09 | 0.84 ± 0.06 | 0.88 ± 0.03 | 0.85 ± 0.06 | Diet x BPA p<.03 |
| | F | 1.13 ± 0.07 | 0.81 ± 0.10 | 1.00 ± 0.10 | 0.84 ± 0.09 | 1.07 ± 0.10 | 1.03 ± 0.05 | |
| **Star** | M | 0.91 ± 0.15 | 0.95 ±0.20 | 0.69 ± 0.14 | 0.90 ±0.14 | 0.89 ±0.07 | 0.98 ± 0.10 | |
| | F | 1.20 ± 0.14 | 0.81 ± 0.10 | 0.98 ± 0.14 | 0.83 ± 0.12 | 1.01 ± 0.09 | 1.06 ± 0.03 | |
| **Cyp11a1** | M | 1.05 ± 0.24 | 1.26 ± 0.19 | 1.24 ± 0.54 | 0.77 ± 0.25 | 1.16 ± 0.41 | 1.13 ± 0.49 | |
| | F | 1.27 ± 0.35 | 1.37 ± 0.40 | 1.80 ± 0.44 | 1.85 ± 0.70 | 1.17 ± 0.27 | 1.67 ± 0.38 | |
| **Inflammation or Oxidative Stress Markers** | | | | | | | | |
| **Cat** | M | 1.03 ± 0.08 | 1.00 ± 0.07 | 0.87 ± 0.07 | 1.19 ± 0.06 | 1.05 ± 0.03 | 1.18 ± 0.06 | Diet p<.005 |
| | F | 1.07 ± 0.04 | 0.98 ± 0.12 | 0.98 ± 0.12 | 1.16 ± 0.10 | 1.13 ± 0.06 | 1.12 ± 0.04 | |
| **Sod1** | M | 1.00 ± 0.07 | 1.00 ± 0.08 | 0.95 ± 0.04 | 1.07 ± 0.10 | 0.97 ± 0.02 | 0.98 ± 0.05 | |
| | F | 1.07 ± 0.05 | 0.91 ± 0.10 | 1.05 ± 0.08 | 1.00 ± 0.09 | 1.10 ± 0.09 | 1.11 ± 0.04 | |
| **Sod2** | M | 0.81 ± 0.08 | 0.69 ± 0.14 | 0.78 ± 0.10 | 0.83 ± 0.12 | 0.81 ± 0.09 | 0.61 ± 0.09 | Sex p<.001 |
| | F | 0.89 ± 0.06 | 0.97 ± 0.14 | 0.98 ± 0.12 | 0.85 ± 0.13 | 1.26 ± 0.12 | 1.27 ± 0.16 | |
| **Glrx** | M | 1.08 ± 0.12 | 1.00 ± 0.11 | 0.90 ± 0.10 | 0.96 ± 0.08 | 0.88 ± 0.07 | 0.87 ± 0.07 | Diet x BPA p<.05 |
| | F | 1.16 ± 0.06 | 0.86 ± 0.11 | 0.88 ± 0.08 | 0.92 ± 0.08 | 1.20 ± 0.08 | 1.30 ± 0.13 | Diet x Sex p<.05 |
| **Aif1** | M | 1.04 ± 0.15 | 1.16 ± 0.18 | 0.91 ± 0.13 | 1.00 ± 0.09 | 0.94 ± 0.11 | 1.17 ± 0.07 | Sex p<.05 |
| | F | 1.14 ± 0.13 | 1.17 ± 0.07 | 1.10 ± 0.14 | 1.26 ± 0.15 | 1.30 ± 0.15 | 1.28 ± 0.05 | |
| **Complement Pathway** | | | | | | | | |
| **C4a** | M | 0.84 ± 0.17 | 1.16 ± 0.10 | 0.93 ± 0.13 | 1.19 ± 0.16 | 1.00 ± 0.09 | 1.34 ± 0.19 | Diet x BPA x Sex p<.05<br>Sex p<.001 |
| | F | 0.85 ± 0.04 | 0.66 ± 0.04 | 0.64 ± 0.06 | 0.77 ± 0.08 | 0.91 ± 0.08 | 0.76 ± 0.04 | |
| **C4b** | M | 0.90 ± 0.08 | 0.91 ± 0.15 | 0.93 ± 0.24 | 0.82 ± 0.10 | 0.81 ± 0.14 | 1.06 ± 0.09 | Sex p<.05 |
| | F | 0.81 ± 0.04 | 0.59 ± 0.09 | 0.80 ± 0.11 | 0.61 ± 0.07 | 0.91 ± 0.11 | 0.74 ± 0.10 | |
| **Apoptosis** | | | | | | | | |
| **Casp3** | M | 0.80 ± 0.06 | 0.81 ± 0.04 | 0.69 ± 0.06 | 0.85 ± 0.05 | 0.74 ± 0.04 | 0.80 ± 0.06 | Diet p<.05 |
| | F | 0.87 ± 0.05 | 0.76 ± 0.08 | 0.74 ± 0.07 | 0.83 ± 0.04 | 0.97 ± 0.05 | 0.97 ± 0.07 | Sex p=.05 |

## Discussion

There were several genes that changed their expression in the mPFC due to perinatal exposure to BPA, HFD and sex. However, there was little consistency between the developmental age, P10, at the end of exposure, and the adult age of P90.

*P10*. Perinatal BPA exposure significantly increased the gene expression of *Erα* in the mPFC of P10 pups. The increase in *Erα* expression seen here is similar but stronger than that reported by Kundakovic et al. (2013) who found a marginally significant U-shaped curvilinear effect. Again, our study included direct BPA administration to the pups from P1-10 which may explain the difference in strength of the results. In the mPFC of P10 rats, the levels of *Erα* mRNA expression should be decreasing (Wilson et al., 2011; Westberry & Wilson, 2012), but it is not clear that the decrease is occurring in our animals. Early developmental alterations in the expression of *Erα* could change the sensitivity of the animals to estrogens, particularly if the increase in *Erα* persists throughout development. This has also been postulated by Cao et al. (2012), who found increases in *Erα* in the hypothalamus due to prenatal BPA exposure.

Gene expression of *Errγ* was also affected by HFD and BPA. ERRγ is an orphan nuclear receptor, which does not bind estrogen (Ijichi et al., 2011). BPA binds strongly to ERRγ (Matsushima et al., 2007) and it is likely that ERRγ is, directly or indirectly, a part of the mechanism of action for BPA effects. When activated, ERRγ is able to modulate estrogen receptor-dependent signaling and even stimulate estrogen response element-mediated transcription without the presence of another ligand (Ijichi et al., 2011). ERRγ has been only recently discovered, and the physiological impact of alterations in its gene expression is unknown. Interestingly, the gene expression levels of *Errγ* were affected by both BPA and HFD. There was a marked increase in expression levels due to HFD that was reduced with BPA

exposure. It is well established that HFD can increase the level of circulating estrogens (Adlercreutz et al., 1994) and the number of ER binding sites in peripheral tissues (Hilakivi-Clarke et al, 1998). However, this is the first reported evidence of an increase in *Errγ* with HFD. It is not known whether there would be any consequences due the increase in gene expression levels of *Errγ* by HFD or the decrease in gene expression levels through the interaction of HFD and BPA exposure. The dearth of information on the function of ERRγ leaves the physiological effect of the changes in its expression unclear.

Expression of pro-inflammatory genes was also affected by BPA and HFD, and as were protein levels of inflammatory markers as described in Chapter 2. Gene expression of *Sod1*, a marker of oxidative stress, was increased significantly with perinatal BPA administration, while gene expression of *Sod2* was increased due to perinatal exposure to HFD. Also in a relatively rare interaction, gene expression of *Glrx*, another gene involved in inflammatory reactions, was increased due to BPA but only in the presence of HFD. The increased expression of *Sod1*, *Sod2,* and *Glrx* in an early developing brain may indicate oxidative stress and inflammation due to maternal HFD and perinatal BPA exposure.

*P90.* Hormone receptors were also altered in adulthood following perinatal BPA and HFD exposure. *Era* increased with HFD and also showed a sex-specific change with BPA. The expression of *Era* increased in females and decreased in males following BPA exposure. Interestingly, *Era* was the only hormone-related gene affected by BPA at both P10 and adulthood. *Era* was significantly increased by BPA in the mPFC of P10 in males and females and in adult females. *Errγ* was also affected at P10 and adulthood, but differentially by BPA and HFD at each time point. At P10, HFD increased the level of *Errγ* expression but in combination with BPA, the levels were significantly reduced. However, there was no effect of BPA on *Errγ*

and HFD are causing a long-lasting change in the expression of *Era* or *Errγ,* which could lead to a differential response to ligands, leading to differences in transcription from early postnatal life into adulthood (Bean et al., 2014).

While not significantly changed at P10, several hormone receptors showed effects of BPA or HFD in adulthood. *Erβ* was decreased with 400 μg/kg/day BPA exposure, but in the presence of HFD, the decrease was abolished. Expression of *Ar* decreased with BPA or HFD exposure, but increased back to control levels with the combination of HFD and BPA. Once again, the changes in the expression of the hormone receptors may suggest a difference in responding to ligands and in transcription.

Genes related to inflammation were also altered in adulthood, but *Glrx* was the only one altered by BPA and HFD at both time points. In adult females, *Glrx* was increased when HFD and BPA were combined. Overexpression of *Glrx* has been found to promote activation of microglia, increasing the release of cytokines (Miller et al., 2016). An increase in the release of cytokines throughout development into adulthood has been associated with altered brain development and behavior (Bilbo & Schwarz, 2009), including depression and schizophrenia (Dantzer et al., 2011; Romero et al., 2007). *Cat* and *Casp3* are also increased following perinatal HFD, while *C4a* is altered by HFD and BPA in a sex-specific manner. Males show an increase in expression of *C4a* following perinatal exposure to both BPA and HFD; however, females generally show a decrease in expression of *C4a*. Notably, neither HFD nor BPA cause an overall increase in the inflammatory markers, which is commonly reported in the literature, but it suggests that not all of the inflammatory pathways are affected by BPA or HFD under these conditions.

To conclude, BPA and HFD have short and long-term effects on the expression of genes related to hormone receptors, steroid synthesis and inflammation. And further, there is evidence of long term effects of BPA on *Erα* in females, HFD at 0 BPA exposure on *Errγ* and females in the HFD group exposed to 400 µg/kg/day BPA on *Glrx*.

# CHAPTER 5: THE NEUROANATOMY OF THE MEDIAL PREFRONTAL CORTEX FOLLOWING PERINATAL BISPHENOL A EXPOSURE

Bisphenol A (BPA) is a synthetic chemical used to create rigid plastics and epoxy resins commonly used in food and drink containers (Vandenberg et al., 2007). BPA can leach out of the product, contaminate the food or drink, and act as an endocrine disruptor by binding to hormone receptors such as ERR γ, ERβ, androgen and thyroid receptors (Takayanagi et al., 2006; Kurosawa et al., 2002; Kritzer & Creutz, 2008; Sohoni & Sumpter, 1998; Moriyama et al., 2002). Inappropriate blocking or activating of hormonal receptors during early brain development could alter normal development and have long-lasting impacts on cognition and behavior.

Previous research in animals has shown effects of BPA on multiple areas of the developing brain, particularly in the subcortical regions where there is a high concentration of hormone receptors (Shugrue et al., 1997). Perinatal BPA exposure has been found to reduce the volume and number of neurons in the sexually dimorphic nucleus in males (McCaffery et al., 2013). Similarly in females, perinatal BPA exposure increases the density of serotonin fibers in the ventral medial nucleus and increases the number of oxytocin cells in the paraventricular nucleus (Adewale et al., 2011). BPA has also been shown to alter the size of the anteroventral periventricular nucleus in both sexes (Arambula et al., 2017). Other areas, particularly the cerebral cortex, have not been thoroughly investigated, despite the presence of steroid hormone receptors during development (Shugrue et al., 1997) and the potential implications of altered development in psychopathologies (Teffer & Semendeferi, 2012). Aside from the work of our laboratory in the mPFC (Sadowski et al., 2014), there are contradictory reports of decreases and increases of dendritic spines in the cerebral cortex in response to perinatal BPA (Kumar & Thakur, 2014; 2017) that are difficult to interpret.

Sadowski et al. (2014) found a significantly higher number of neurons and glia in the deep layers of the medial prefrontal cortex (mPFC) in adult male rats, but not in female rats, following perinatal BPA exposure. This finding could have broad implications for later behavior since the prefrontal cortex (PFC) is necessary for higher-order cognition and alterations in development of the PFC are involved in schizophrenia, depression, and autism (Zhou et al., 2015; Price & Drevets, 2012; Teffer & Semendeferi, 2012). Autism is a developmental disorder that is more likely to occur in males than females, and the cortex of young males with autism often has a higher number of neurons, especially in the prefrontal cortex (Courchesne et al., 2007; Edmonson et al., 2014). The higher number of neurons and glia found in Sadowski et al.(2014) is male-specific and follows a similar pattern. In addition to cell number, changes in the number of synapses in the PFC have also been implicated in psychopathologies (Tang et al., 2014). The Sadowski et al.(2014) study did not assess synaptic changes within the mPFC, and we hypothesize that the number of synapses would mirror the increase in the number of neurons. The current study aims to expand the previous research by investigating the long-term effects of perinatal BPA exposure on the number of synapses and the number of synapses per neuron in the mPFC.

In addition to neurons, the Sadowski et al. (2014) study found a higher overall number of glia in the mPFC following perinatal BPA exposure. Using a Nissl stain, the specific subtype of glia could not be identified. However, we have found that both microglia and astrocytes are impacted by BPA exposure during adolescence (Wise et al., 2016). Perinatal BPA has been found to increase inflammation and the mRNA expression of microglial markers in mouse brains (Luo et al., 2014), suggesting an increase in activation and perhaps proliferation; therefore, we hypothesize that the higher number of glia may be due to a higher number of microglia. The

current study further expands the previous research by quantifying the number and characterizing the morphology of microglia in the adult mPFC following perinatal BPA exposure.

## Methods

*Breeding*

Male and female Long Evans hooded rats purchased from Harlan Laboratories (now Envigo, Indianapolis, IN, USA) were housed in our vivarium for at least one week before being paired for breeding. They were kept on a 12:12 light/dark cycle and were allowed food and water ad libitum. Precautions were taken to reduce the environmental exposure to endocrine disruptors. All animals were housed in BPA-free polysulfone cages and water was reverse osmosis filtered in glass bottles. Animals were fed OpenSource Diets product number D10012G (Research Diets Inc., New Brunswick, NJ, USA). OpenSource Diets D10012G was the control diet as mentioned in previous chapters. For the neuroanatomical portion of this study, only the control diet animals were assessed upon recommendation from the Children's Centers' external review board.

Breeding pairs were placed in suspended wire bottom cages and checked daily for the presence of sperm plugs. The day a sperm plug was detected was recorded as gestational day (G) 0 and the dams were singly housed in a polysulfone shoebox cage. The breeding animals were paired for a maximum of six nights. If no sperm plug was detected after six nights, the male was removed and another male introduced. From the first day of pregnancy, each female was assigned to one of three groups. The numbers of litters for each BPA exposures are: 0 µg BPA/kg = 10; 40 µg BPA/kg = 11; 400 µg BPA/kg = 12. As this is a portion of a larger study, the breeding was done over several months, resulting in six cohorts of animals.

*BPA Dosing*

BPA (received from the Environmental Protection Agency (EPA), 99% purity) was suspended in tocopherol-stripped corn oil at 0, 0.1 mg BPA/ml, or 1.0 mg BPA/ml in order to administer 0 (control), 40 µg BPA/kg , or 400 µg BPA/kg respectively. To dose the adult dams, the required amount (0.4 µl/g body weight) was pipetted onto ½ of a cookie (Newman's Own organic alphabet cookie, vanilla flavor) and given to the animals. The animals readily consumed the cookie making this route of exposure non-stressful and similar to human ingestion of BPA. On G0 and G1, the dams were given ½ of a cookie with 0.4 µl/g tocopherol-stripped corn oil. Starting on G2 through parturition, the dams were given the cookie with the assigned BPA dose. The day of birth was recorded as P0 and the litters were not disturbed. Then daily from P1-10, each pup was individually dosed via pipetting the assigned solution (same as dam) directly into its mouth because lactational transfer of BPA is very low in rats (Doerge et al., 2010). Brain development during the first ten postnatal days in a rat is roughly equivalent to the brain development that occurs during the third trimester of gestation in a human (Romjin et al., 1991), so we extended our dosing regimen to include the first ten postnatal days. At P2, the litters were culled to a maximum of 10 pups, and animals were weaned at P25 and housed in same-sex dyads or triads. Only one male and one female was used from each litter to limit within litter confounds. These animals were subjected to periadolescent and social recognition behavioral tasks as reported in chapter 3.

*Histology*

Following the behavioral tasks in adulthood (P90), the animals were deeply anesthetized with Fatal-Plus (Vortech Pharmaceuticlas LTD, Dearborn, MI, USA), and the brains were fixed

via transcardiac perfusion using .1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in .1 M PBS. The brain was immediately removed and placed in 4% paraformaldehyde in .1 M PBS for 24 hours, and then transferred to 30% sucrose in .1 M PBS solution for 48 hours. Before slicing, each brain was coded with a unique number to keep the experimenters blind to the sex or BPA exposure of each animal. 40 μm coronal slices were taken using a freezing microtome. Every sixth slice (240 μm) was plated onto a charged slide and allowed to dry for 24 hours. The slices were then stained with methylene blue/azure II and coverslipped. Remaining adjacent slices were placed in storage solution (30% glycerol, 30% ethylene glycol, 30% distilled water, 10% .1M PBS) and held in a -20 C freezer until immunohistochemical staining.

*Immunohistochemistry*

The adjacent 40 μm slices were removed from the storage solution and rinsed three times in tris-buffered saline (TBS; pH 7.6). The slices were submerged in a blocking solution (TBS containing 1% hydrogen peroxide, 20% normal goat serum [NGS] and 1% bovine serum albumin [BSA]) for 30 mins and then incubated in primary antibody (anti IBA1 rabbit, Wako Chemicals USA, Richmond, VA, USA [microglia] or anti-synaptophysin mouse, MilliporeSigma, St. Louis, MO, USA [synapses]) for 48 hours. The primary antibody concentration for both anti IBA1 and anti synaptophysin was 1:5000 and made in TBS containing 2% NGS and 0.3% Triton-X-100 (TTG). After primary incubation, the slices were rinsed three times in TTG, and incubated in secondary antibody (5μg/ml; biotinylated goat, anti-rabbit IgG [microglia] or biotinylated goat, anti-mouse IgG [synapses]) for 90 mins. The slices were then rinsed twice in TTG, twice in TBS, and then incubated in avidin biotin complex (ABC) solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for one

hour. The slices were then rinsed three times in TBS and submerged in DAB solution (Sigma-Aldrich fast tabs, St. Louis, MO, USA) for two minutes followed by multiple rinses in TBS. The slices were then mounted on charged slides, allowed to dry for 24 hours, dehydrated and coverslipped.

*Cellular quantification*

*Volume of the mPFC*

The slices stained with methylene blue/azure II were used to quantify volume of the mPFC as well as the number of neurons and glia. Both the prelimbic (PL) and infralimbic (IL) portions of the mPFC were parcellated using distinguishing cytoarchitecture as outlined in Markham et al. (2007) (Fig. 1A). The dorsal boundary of the PL is identified by a widening of Layer V and an altered trajectory of cell orientation. The ventral boundary of the IL is identified by a loss of the laminar organization of the cells. Parcellation of the anterior mPFC began when the lamination pattern was clearly present and the posterior boundary of the mPFC was marked with the appearance of the genu of the corpus callosum. The area and thickness of the layers II-VI were used to calculate the volume of the mPFC for all of the cellular calculations. The volume of Layer I was included for synapse number when analyzing synaptophysin. The volume of the subcortical white matter under the anterior to posterior sections containing the mPFC was also measured.

*Neurons and glia*

The numbers of neurons and glia were stereologically counted in the methylene blue/azure II stained section with the optical disector as in Markham et al. (2007) and Wise et al. (2016) with StereoInvestigator software (Microbrightfield, Williston, VT, USA). The software

randomly selects areas to analyze based upon the parcellated region of interest. A 35 µm x 35 µm counting frame with "inclusion" and "exclusion" lines were used. Any cell that was inside the counting frame or touching an inclusion line was counted. Any cell that was outside the frame or touching an exclusion line was not counted. The counting frame also had a z-axis depth of 12 µm with 1 µm guard zones on the top and bottom. The bottom of each cell had to be in focus within the 10 µm (12 µm minus 1 µm guard zone on top and bottom) counting frame in order to be counted. This protects against the bias of counting larger cells more often than smaller cells.

When using methylene blue/azure II, neurons and glia are distinguishable based upon color and shape. Neuronal cell bodies stain a dark blue and are larger in size than the glia. Glial cell bodies stain a light blue/turquoise color, are smaller than neurons, and have an amorphous shape (Fig. 1B). A minimum of 400 neurons and 200 glia were counted from the upper and from the lower layers of each brain. Separately for neurons and glia, the density (cells per volume) was multiplied by the total volume of the mPFC to find the total number neurons and glia in the mPFC. The numbers of males and females, respectively, for each BPA exposures are: 0 µg BPA/kg = 10, 9; 40 µg BPA/kg = 11, 9; 400 µg BPA/kg = 12, 12.

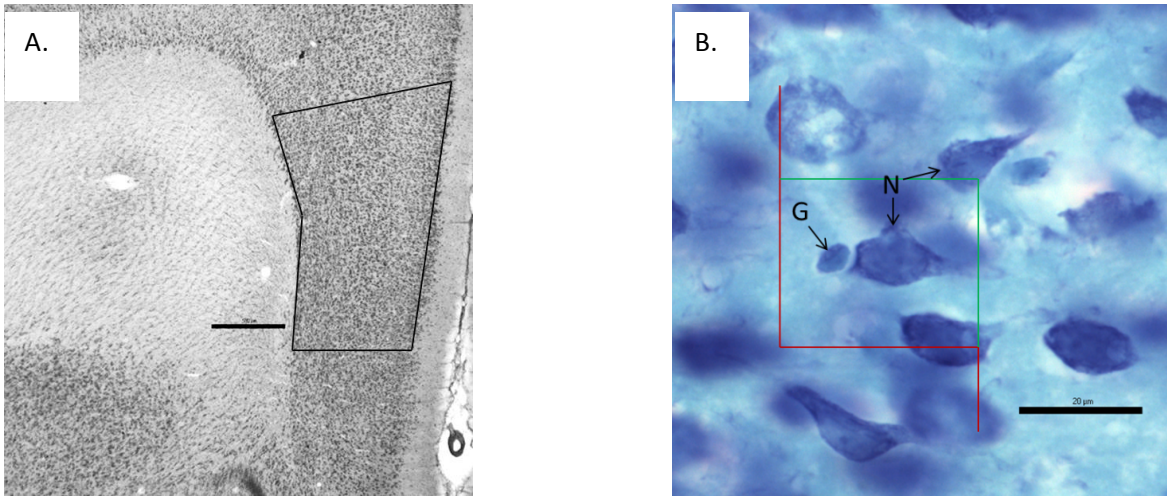



Figure 5.1. A. A coronal view of the rat mPFC. The tracing encompasses Layers II-VI of the PL and IL of the mPFC bordered by Layer I and the white matter. Scale bar = 500 µm. B. An example of the counting frame in the optical disector with red exclusion lines and green inclusion lines and the neurons (N) and glia (G) that may be counted surrounding the frame. Scale bar = 20 µm.

*Quantification of synapses*

Synaptophysin is an integral protein in the SNARE complex at each synaptic cleft and is commonly used to label synapses (Calhoun et al., 1996). The same stereological methods as outlined above were used to calculate the number of synapses in Layers I-VI in the mPFC using the immunostained slices. To accommodate their smaller size, the counting frame size was reduced to 4 µm x 4 µm with a 6 µm depth on the z-axis (including 1 µm top and 1 µm bottom guard zones; Fig. 2). The density of synapses was multiplied by the volume of the region in order to obtain the total number of synapses in the mPFC. In addition, for each animal, the number of synapses was divided by the total number of neurons to estimate the average number of synapses per neuron. The numbers of males and females, respectively, for each BPA exposures are: 0 µg BPA/kg = 8, 8; 40 µg BPA/kg = 11, 8; 400 µg BPA/kg = 9, 11.

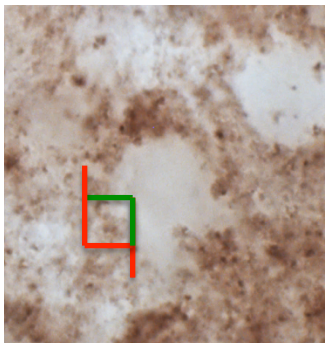
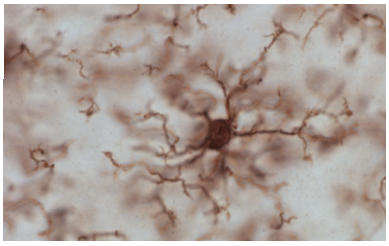


Figure 5.2. Immunostained synaptophysin at high magnification with a 4 x 4 µm counting frame including exclusion (red) and inclusion (green) lines.

*Microglia Quantification and Classification*

The optical dissector was also used to count the number of microglia in the mPFC in the slices immunostained for IBA1. To account for a smaller number of cells, the counting frame dimensions were enlarged to 75 µm x 75 µm with an 8 µm depth on the z-axis (including 1 µm top and 1 µm bottom guard zones). Every microglia cell body was considered for inclusion regardless of morphological state. The density of microglia was multiplied by the volume of the layers II to VI to quantify the total number of microglia in the mPFC. Further, the morphological state of each microglia was classified as a ramified, primed, reactive, or amoeboid phenotype as described in Torres-Platas et al. (2014). An example of a ramified and amoeboid microglia are shown in Figure 3. The numbers of males and females, respectively, for each BPA exposures are: 0 µg BPA/kg = 9, 8; 40 µg BPA/kg = 11, 10; 400 µg BPA/kg = 12, 10.

A. 

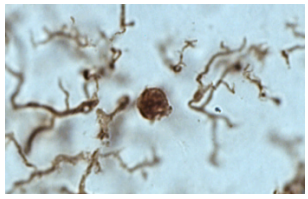
B. 

Figure 5.3. High magnification images of ramified (A) and amoeboid (B) microglia in the mPFC of rats exposed to perinatal BPA.

*Statistics*

A univariate analysis of variance (ANOVA) with BPA exposure as the independent factor and cohort as a cofactor was used to analyze the neuroanatomical measures (IBM SPSS Statistics version 24, New York, NY, USA). The assumptions of ANOVA were met: the data were normal, independent and identically distributed. A p-value of $p<.05$ defined a significant difference between groups. Since previous literature has shown males and females to react differently to BPA exposure (Sadowski et al, 2014), each sex was analyzed separately.

## Results

*Neurons and glia*

The number of neurons was not significantly altered by perinatal BPA exposure in males, $F(2,32)=1.01$, $p>.05$, or females, $F(2,29)=.002$, $p>.05$ (Fig. 5.4). The males exposed to 400 µg/kg/BPA/day had a non-significant 10% increase in the number of neurons in the mPFC compared to controls. This pattern is closely following previous data from our laboratory that showed a significant 15% increase in the number of neurons only in males exposed to 400 µg/kg/BPA/day (Sadowski et al., 2014).

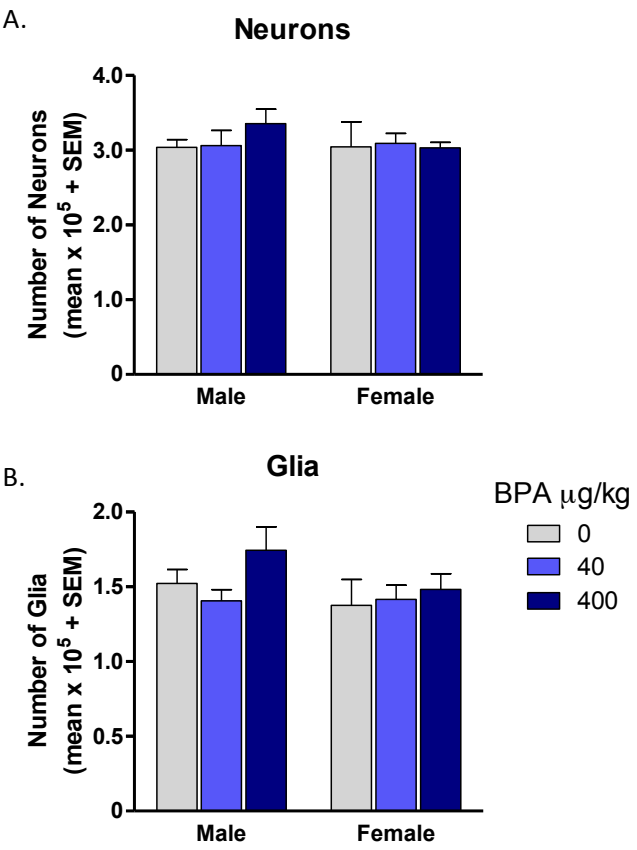



Figure 5.4. The number of neurons (A) and glia (B) in the mPFC in adult male and female rats following perinatal BPA exposure.

The number of glia was also not significantly altered by perinatal BPA exposure in males, $F(2,32)=2.14$, $p>.05$ or females, $F(2,29)=0.36$, $p>.05$ (Fig. 5.4). Similar to neurons, the number of glia in males exposed to 400 μg/kg/BPA/day had a non-significant 14% increase compared to controls. Once again, this pattern follows previously published data showing a significant 19% increase in the number of glia only in males exposed to 400 μg/kg/BPA/day (Sadowski et al., 2014).

There was a marginal increase in the volume of the white matter in males, $F(2,32)=2.76$, $p=.08$ (Fig. 5.5) with perinatal BPA exposure. Females did not have a significant change in the

volume of white matter following BPA exposure, F(2,29)=0.66, p>.05. This non-significant increase in white matter volume was also observed in Sadowski et al. (2014), but has a greater magnitude in the current study.

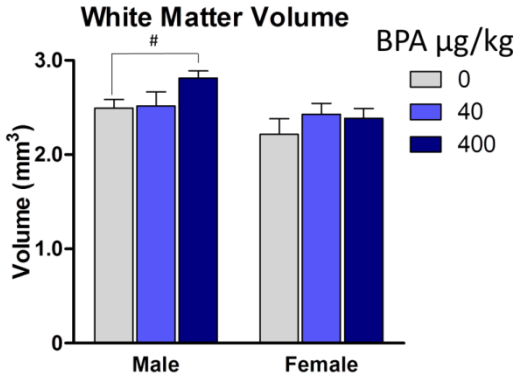



Figure 5.5. Volume of the subcortical white matter in adult male and female animals following perinatal BPA exposure. #p=.08.

*Synapses*

The number of synapses labelled with synaptophysin was not significantly altered by perinatal BPA exposure in males, F(2,27)=0.43, p>.05, or in females, F(2,24)=0.39, p>.05 (Fig. 5.6). There was a near significant effect of BPA exposure on the number of synapses per neuron in males, F(2,27)=3.53, p=.051, but a Dunnett's posthoc revealed neither dose to be significantly different from control. The number of synapses per neuron was not significantly altered by BPA exposure in males in females, F(2,24)=0.08, p>.05 (Fig. 5.6).

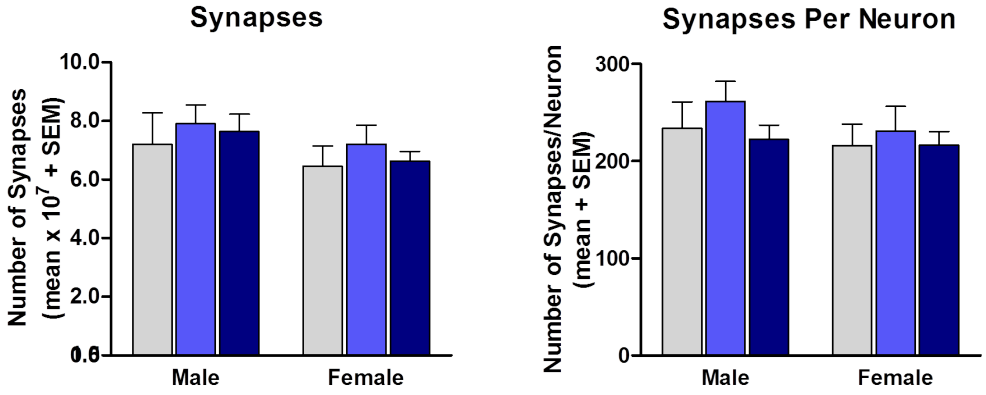



Figure 5.6. The number of synaptophysin-immunoreactive synapses and average number of synapses per neuron in the mPFC of adult male and female rats following perinatal BPA exposure.

*Microglia*

The total number of microglia was not significantly altered by perinatal BPA exposure in males, $F(2,31)=1.15$, $p>.05$, or females, $F(2,26)=0.87$, $p>.05$ (Fig. 5.6). The morphology of microglia was rarely affected by perinatal BPA exposure in males or females (Table 5.1). In males, there was no significant change in the percent of ramified, $F(2,31)=0.21$, $p>.05$, primed $F(2,31)=0.18$, $p>.05$, activated $F(2,31)=1.07$, $p>.05$, or ameboid, $F(2,31)=0.17$, $p>.05$ microglia. In females, there was no significant change in the percent of ramified, $F(2,26)=0.32$, $p>.05$, primed, $F(2,26)=0.32$, $p>.05$, or activated, $F(2,26)=0.62$, $p>.05$, microglia. Females showed a significant decrease in the percent of microglia in the ameboid state, $F(2,26)=3.51$, $p=.048$, following perinatal BPA exposure. However, caution should be used in interpreting the results, given that less than 1% of microglia were in the ameboid state.

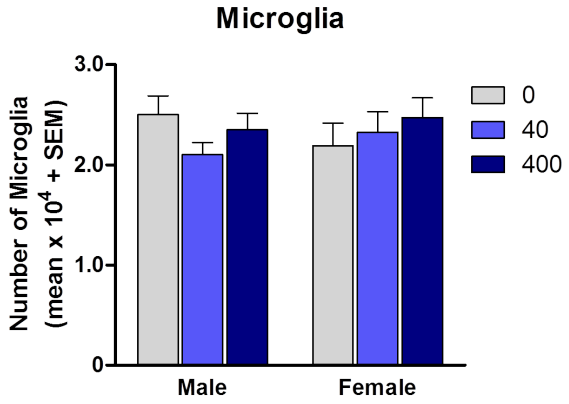



Figure 5.7. The number of microglia in the mPFC following perinatal BPA exposure.

Table 5.1. Percent of microglia in each morphological phenotype (Mean ± SEM)

| | | BPA Exposure (µg/kg/day) | | |
|---|---|---|---|---|
| | Sex | 0 | 40 | 400 |
| Ramified | M | 71.8 ± 7.0 | 74.4 ± 6.1 | 72.9 ± 4.8 |
| | F | 72.6 ± 9.2 | 63.8 ± 8.5 | 68.6 ± 7.2 |
| Primed | M | 27.6 ± 7.0 | 25.3 ± 6.0 | 25.8 ± 4.0 |
| | F | 26.4 ± 8.9 | 34.9 ± 8.0 | 31.0 ± 7.1 |
| Activated | M | 1.0 ± 0.01 | 0.07 ± 0.10 | 0.90 ± 0.80 |
| | F | 0.04 ± 0.04 | 1.3 ± 1.20 | 0.2 ± 0.20 |
| Amoeboid | M | 0.4 ± .002 | 0.3 ± .001 | 0.3 ± .002 |
| | F | 0.9 ± 0.50* | 0.0 ± 0.00* | 0.1 ± 0.10* |

*p<.05

## Discussion

Perinatal BPA exposure did not have a significant long-term effect on the number of neurons, glia, synapses or microglia in the mPFC of adult animals. In prior research, the number of neurons increased by 15% and the number of glia increased by 19% in the mPFC of males only exposed to 400 µg/kg/day perinatal BPA (Sadowski et al., 2014). In the current study, there was a similar number of subjects (n=9-12) as the Sadowski et al.study (n=8-13). Here, there was a numerically smaller (by 5%), non-significant increase for both types of cells in males: 10%

increase in neurons and 14% in glia in the 400 µg/kg/day exposure group. While the increases in the current study are not statistically significant, the patterns of change are strikingly similar to the findings of Sadowski et al. (2014). The effect of BPA on the number of neurons and glia in the mPFC may be a small effect, and thus, difficult to detect with this sample size. The effect may be significant with increased power.

There were differences between the studies that could contribute to a difference in the number of cells in the mPFC of the animals. First, the animals all were assessed for play behavior with a same-sex non-cage mate four times during adolescence (P26-40) (Chapter 3). Adolescence is known to be a time of restructuring in the brain, including the mPFC (Juraska and Willing, 2017). It is possible that the play behavior during this time was a form of enrichment to the periadolescent animals, resulting in a permanent neuroanatomical change that interacted with the effect of BPA. Environmental enrichment during adolescence has been found to alter neuroanatomical measures in the mPFC of rats (Mychasiuk et al., 2014) although changes in neuronal pruning due to enrichment have not been explored. Second, the food that the dams ate during BPA exposure was from two different sources (Envigo Teklad 2020X and Research Diets Open Source D10012G). While comparable on the percent of macronutrients, the protein sources in the Sadowski et al.(2014) study (Envigo Teklad 2020X) were corn and wheat, whereas the protein sources in the current study (Research Diets Open Source D10012G) was casein, a protein commonly found in milk. Given the importance of protein in the maternal diet during gestation for optimal brain development (Morgane et al., 1978), the different protein sources during neural development could potentially interact with the BPA exposure and lead to the differences in neuron number between the studies.

The current study did not find any long-lasting effects of perinatal BPA exposure on the number of synaptophysin-immunoreactive synapses in the mPFC of adult male or female rats. Our hypothesis that the number of synapses would increase along with the number of neurons in male animals exposed to 400 μg/kg/day BPA was not supported. 500 μg/kg/day prenatal BPA exposure has been found to decrease the mRNA levels of synaptophysin in the hippocampus of male mice at P21, but not the protein level as measured by western blot (Wang et al., 2014). Furthermore, low circulating levels of BPA in a non-human primate also decreased the number of spine synapses in the dorsolateral PFC in males; yet, four weeks after the removal of the osmotic pump, the number of spine synapses had significantly increased towards complete recovery (Elsworth et al., 2015); although, it should be noted that the time of exposure was during young adulthood, not perinatal. Taken together, these findings suggest that BPA exposure may have a transient effect on the number of synapses, but is not causing a lasting loss of synapses. However, longer-term exposure to BPA, as is the case for the human population, may not allow recovery of synapses.

Similar to the number of synapses, the current study did not find a significant difference in the number of microglia in the mPFC following perinatal BPA exposure. Likewise, there were no significant differences in the percent of cells in the ramified, primed or activated morphological states. Females had a significant decrease in the percentage of amoeboid microglia. However, less than 1% of the microglia were in the amoeboid state and this sample size may be too small to be reliable. Similar to the data on synapses, previous work on immune activation in the PFC following perinatal BPA exposure shows an increase in mRNA and protein levels of proinflammatory factors, IL-6 and TNF-α, and the microglia marker, IBA1, (Luo et al., 2014) when the animals are assessed while still exposed to BPA. Given that microglia have a

complete population turnover about every 95 days (Askew et al., 2017), perhaps it is not surprising that perinatal BPA exposure would not cause a long-term effect on the number or morphological state of microglia in the mPFC in adulthood.

In conclusion, the current study found a non-significant numerical increase in the number of neurons and glia in the mPFC of adult male animals following perinatal BPA exposure. The pattern of the increase is very similar to the significant increases found in Sadowski et al. (2014). Furthermore, there are no differences in the number of synapses, the number of synapses per neuron or in the number of microglia. Previous work has found that many of these measurements can show effect immediately after BPA exposure, but the current study suggests that if there are long-term neuroanatomical effects of perinatal BPA in the mPFC, they are not robust.

# CHAPTER 6: GENERAL CONCLUSIONS

The general conclusion to these studies is that perinatal exposures to BPA and HFD are producing lasting effects; however, some of the effects are subtle. Significant alterations were found in maternal care, inflammatory markers, gene expression at P10 and P90, play behavior and the elevated plus maze. Perinatal BPA and HFD did not produce additive effects in this study. Each had separate effects and the two rarely interacted.

Perinatal BPA caused most of the significant results in this study. Specifically, 400 μg/kg/day perinatal BPA exposure caused the majority of the significant effects compared to control, but the 40 μg/kg/day group often produced effects between the 0 and 400 μg/kg/day, but usually not significantly different from control. Four hundred μg/kg of oral BPA exposure has been found to produce a urinary concentration in non-human primates similar to that found in humans (Taylor et al., 2011). Thus, the effects from the 400 μg/kg/day perinatal BPA exposure found in the current study are likely to be relevant to human exposure.

Perinatal HFD produced few effects and was not obesogenic suggesting that short-term exposure to a 45% HFD may not be sufficient to produce significant metabolic effects on the offspring. However, continued exposure to a HFD may be more likely lead to metabolic, behavioral and cognitive effects as reported in previous literature (as reviewed in Bilbo & Tsang, 2010). Additionally, the dams were not obese at the beginning of the study, which also may have led to minimal effects on the offspring.

In terms of translation to humans, it should be noted that we restricted the exposure period to gestation and the first ten postnatal days to encompass and focus on the brain development that occurs during human gestation. In many humans, the exposure to BPA and

HFD likely continues throughout the lifespan. The impact of BPA and HFD at different times is likely to have different effects. Similarly, the changes in inflammation during brain development may lead to an enhanced activation in the event of an immune challenge occurring later in life. Further research is necessary to determine the full impact of life long exposures to BPA and HFD.

The current work focused on the mPFC, as it is involved in executive functioning, cognition, and has been implicated in psychopathologies. Another aspect of this work is the lack of statistical significance, but also the similarity, of the neuroanatomy results in the mPFC compared to the previous work from our laboratory. The higher number of neurons and glia in this study was not significant, but the pattern closely followed the pattern found by Sadowski et al. (2014) as did the volume of the white matter which was marginally significant here but not in the Sadowski et al. study. Both studies had a comparable number of subjects, 9-12 in the current study and 8-13 in the Sadowski et al. study (2014). However, the Sadowski et al. (2014) paper reported a significant cohort effect, which was not found here, which suggests that there were some unaccounted for differences between the studies. Also the diets differed between the studies, in particular the source of protein. It is unclear if this could change the amount of influence that BPA has on the developing cortex.

Finally, the current set of studies encompassed a variety of behaviors, as well as genetic and neuroanatomical analyses, but there are other end points that could be investigated. While we found effects in many of our analyses, perinatal BPA and HFD may have further implications for other types of behavior and areas of the brain. Even our fairly modest findings indicate that exposure to BPA during human gestation should be limited if possible.

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